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WO 9605303A1

INTERNATIONAL APPLICATION PUBLISHED UNDER T.

(51) International Patent Classification ⁶:
C12N 15/12, C07K 14/705, A61K 39/395,
C12N 15/11, A61K 38/17, C07K 16/28.

(11) Internati nal Publication Number:

WO 96/05303

C12N 15/11, A61K 38/17, C07K 16/28, G01N 33/68

(4)

A1

(43) International Publication Date: 22 February 1996 (22.02.96)

(21) International Application Number:

PCT/US95/09636

(22) International Filing Date:

9 August 1995 (09.08.95)

9.08.95)

6 (81) Designated States: AU, CA, IP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(30) Priority Data:

289,699

12 August 1994 (12.08.94)

US

(71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDA-TION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).

(72) Inventors: FUKUDOME, Kenji; 13125 West Park Place, Oklahoma City, OK 73142 (US). ESMON, Charles, T.; 5800 North Stonewall, Oklahoma City, OK 73111 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

(57) Abstract

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca²⁺ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role f protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

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CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

Background of the Invention

The present invention is generally in the area of cloning, expression, and regulation of an endothelial cell protein C/activated protein C receptor.

5 Protein C plays a major role in the regulation of blood coagulation. Patients deficient in protein C usually exhibit life threatening thrombotic-complications in infancy (Seligsohn et al., (1984) N. Engl. J. Med. 310, 10 559-562; Esmon, (1992) Trends Cardiovasc. Med. 2, 214-220) that are corrected by protein C administration (Dreyfus et al., (1991) N. Engl. J. Med. 325, 1565-1568). In addition, activated protein C (APC) can prevent the lethal effects of 15 E. coli in baboon models of gram negative sepsis (Taylor et al., (1987) J. Clin. Invest. 79; U.S. Patent No. 5,009,889 to Taylor and Esmon) and preliminary clinical results suggest that protein C is effective in treating certain forms of human 20 septic shock (Gerson et al., (1993) Pediatrics 91, 418-422). These results suggest that protein C may both control coagulation and influence inflammation. Indeed, inhibition of protein S, an 25 important component of the protein C pathway, exacerbates the response of primates to sublethal levels of E. coli and augments the appearance of TNF in the circulation (Taylor et al., (1991) Blood 78. 357-363). The mechanisms involved in controlling the inflammatory response remain unknown.

Protein C is activated when thrombin, the terminal enzyme of the coagulation system, binds to an endothelial cell surface protein, thrombomodulin (Esmon, (1989) <u>J. Biol. Chem.</u> 264, 4743-4746; Dittman and Majerus, (1990) <u>Blood</u> 75, 329-336;

Dittman, (1991) Trends Cardiovasc. Med. 1, 331-336). In cell culture, thrombomodulin transcription is blocked by exposure of endothelial cells to tumor necrosis factor (TNF) (Conway and Rosenberg, (1988) Mol. Cell. Biol. 8, 5588-5592) and thrombomodulin activity and antigen are subsequently internalized and degraded (Lentz et al., (1991) Blood 77, 543-550, Moore, K.L., et.al., (1989) Blood 73, 159-165). In addition, C4bBP, 10 a.regulatory protein of the complement system, binds protein S to form a complex that is functionally inactive in supporting APC anticoagulant activity in vitro (Dahlbäck, (1986) J. Biol. Chem. 261, 12022-12027) and in vivo (Taylor, et al., 1991). Furthermore, C4bBP behaves as an acute phase reactant (Dahlbäck, (1991) Thromb. Haemostas. 66, 49-61). Thus, proteins of this pathway not only appear to regulate inflammation, but they also interact with 20 components that regulate inflammation, and they themselves are subject to down regulation by inflammatory mediators repulsed to pathway in regulating the host response to . 25 inflammation and the critical role of the pathway which in controlling blood coagulation, it is important to identify and characterize all of the components that interact with the system. This is especially true since the molecular basis of the anti-inflammatory effects of the protein C pathway .30⋅ components have yet to be elucidated at the molecular levely and the analysis of which is therefore an object of the present invention to provide a cellular receptor for protein C and activated protein C. Missa Born Long It is a further object of the present w invention to provide nucleotide sequences encoding

the cellular receptor and amino acid characterization of the receptor which allows expression of recombinant native and modified forms of the receptor. Tyes the community

5 It is another object of the present invention to provide methods of modulating the inflammatory response involving protein C and activated protein C. 14 No. 124 Activated Protein C.

Summary of the Invention 10 An endothelial cell protein C binding protein (referred to herein as mEPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM) and is calcium dependent. The message and binding function of the receptor are both down 20 regulated by cytokines such as TNF. These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of EPCR can be enhanced by

overexpressing the EPCR in endothelium that could the commence of the state of th

be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

Brief Description of the Drawings Figures 1A, 1B and 1C are flow cytometric analyses of F1-APC (fluorescent labelled activated protein C) binding to HUVEC (human umbilical vein endothelial cells). Figure 1A is a graph of cell number versus log of fluorescence intensity, demonstrating F1-APC binding to HUVEC. HUVEC (1 x 10 105) were incubated at room temperature without (dotted line) or with 160 nM of F1-APC (solid line) in the presence of 1.3 mM CaCl2. After washing, bound APC was analyzed by flow cytometry. Figure 1B is a graph of fluorescence intensity versus F1-APC concentration (nM) demonstrating the concentration dependence of F1-APC binding to HUVEC. HUVEC were incubated with F1-APC in the absence (open circles) or presence of 1.3 mM CaCl. (closed circles) and binding was measured as in A. Mean channel fluorescence intensity is plotted for each F1-APC concentration (between 0 and 800 nM). Figure 1C is a graph of the percent of mean fluorescence versus unlabeled protein concentration - (μg/ml), demonstrating the effects of unlabeled proteins on F1-APC binding to HUVEC. F1-APC binding to HUVEC was carried out in the presence of the indicated concentrations (between 0 and 100 μ g/ml) of unlabeled APC, protein C, protein S, factor X and Xa or recombinant Gla-domainless

Figures 2A, 2B, 2C and 2D are graphs of ¹²⁵I-APC Binding to HUVEC Monolayers. Figure 2A is a graph of the bound APC (cpm x 10⁻³) versus time (min), showing the time course of ¹²⁵I-APC binding to HUVEC. HUVEC monolayers (1.2 x 10⁵ cells) were incubated at 4°C with 32 nM (filled squares) or 8

· 30 protein C (rGDPC).

... nM (open squares) 125 I -APC. At the indicated times, cells were washed and bound radioactivity was measured. Figure 2B is a graph of bound APC (cpm x 10-1) versus unlabeled protein (nM) demonstrating 5 the effects of unlabeled APC and rGDPC on 125I-APC binding to HUVEC. HUVEC were incubated at 4°C for one hour with 125 I-labeled APC in the presence of the indicated concentrations (between 01 and approximately 1000 nM) of unlabeled APC (open 10 circles) or rGDPC (closed circles). After washing, bound radioactivity was measured. Figure 2C is a graph of bound APC (fmol/well) versus free APC (nM) APC binding to HUVEC. Monolayers of HUVEC were incubated with the concentration demonstrating the concentration dependence of 125 Iindicated as described above. Specific binding was Figure 2D is a determined as described below. Scatchard analysis of 1251-APC binding to HUVEC. Each value was calculated from the data shown in Figure 2C. 20

Figures 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the absence (dotted lines) or presence of 1.3 mM CaCl₂ (solid lines).

Figures 4a-4c are the predicted protein structure of EPCR based on nucleotide sequence (SEQ ID NO. 1), predicted amino acid sequence (SEQ ID NO. 2) and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated with the solid bars.

Figures 5a-5b are a comparison of the amino acid sequence of EPCR to the amino acid sequences of other members of the CD1 family and CCD41. The EPCR sequence is shown in the first line and

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compared to murine CCD41 (second line), human CD1d (third line) and murine CD1.2 (fourth line): Identities, with ERCR are indicated by open boxes. Residues that are conserved between EPCR and all of 5 the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single in prasterisk; promp program and for some of or Figure 6, is a comparison of the amino 10 acid sequence of human EPCR (first line) to the amino acid sequence of murine EPCR (second line). Identities are indicated by lines. Similarities refare indicated with dots. The land of the second of the to messes while it elements on the least on a despe Detailed Description of the Invention 15 Cloning and Characterization of EPCR. Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably (Kd = 30 nM, 7000 sites per cell) in a Ca2 dependent fashion. FL-APC 20, binding to various human cell lines were examined, and found that the binding was HUVEC specific. human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990) Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into 30 293T cells, using the method of Kaisho et al., (1994) Proc. Natl.: Acad. Sci. (USA) 91, 5325. FL-APC binding was analyzed on a flow cytometer. One of eight subpools gave a positive signal. subpool was divided into 20 subpools and 35, con rescreened and After three rounds of enrichment, one Management of the control of the con

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carries a 1.3 kb insert When transfected into 293T cells, this clone was capable of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface

that coded for a type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endethelial cell protein C receptor (EPCR) function and message are both down regulated

by exposure of endothelium to TNF. Identification of EPCR as a member of the CDI/MHC superfamily provides insights into the role of this receptor

for protein C in regulating the inflammatory response.

Materials and Methods

Protein Preparation

Human protein C (Esmon et al., (1993)

Meths. Enzymol. 222, 359-385), APC (Esmon et al., 1993); recombinant gla domainless protein C (rGDPC) (Rezaie et al., (1992) J. Biol: Chem. 267, 11701-11704), protein S (Taylor et al., 1991),

factor X and tactor Xa (Le Bonniec et al., (1992)

25 <u>J. Biol. Chem.</u> 267, 6970-6976) were prepared as described in the cited publications.

Selective labeling of the active site of APC with fluorescein was performed by the method of Bock (Bock, P.E. (1988) Biochemistry 27,

30 6633-6639). In brief, N°-22 200 200 μM)
[(acetylthio)acetyl] D-Phe-Pro-Arg-CH₂Cl (200 μM)
was reacted with 40 μM. APC for 1 hour at room
temperature. After dialysis, the covalently
modified APC was incubated at room temperature for

one hour with 200 µM 5-(iódoacétamido)fluorescein (Molecular Probes). Free fluorescein was removed by gel filtration on a PD-10 column (Pharmacia).

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With this method, each molecule of fluoresceinated APC (FI-APC) contains a single dye at the active site and hence all of the fluorescent molecules behave identically.

Iodogen (Pierce) was used to radiolabel APC with Na[125] (Amersham) according to the manufacture's protocol in the presence of 5 mM CaCl₂. Free 125I was removed by gel filtration on a PD-10 column. The specific activity of the 125I-APC was 1 x 104 cpm/ng protein.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical-cords by collagenase treatment and cultured in medium 199 15 containing 15% fetal bovine serum, 10 μ g/ml heparin, and 0.5% endothelial cell growth supplement prepared from bovine brain extract (Maciag at al., (1979) Proc. Natl. Acad. Sci. (USA) 76, 5674-5678). HOS (ATCC CRL 1543), HEp-2 (ATCC CCL 23) and 293 cells (ATCC CRL 1573) transformed 20 with SV40 large T antigen (293T, a gift from Dr. Kenji Oritani) were maintained in Earl's MEM supplemented with 10% fetal bovine serum. The human lymphocyte cell lines, Jurkat, MOLT3 (ATCC CRL 1552), Jijoye (ATCC CCL 87), Raji (ATCC CCL 86), U-937 (ATCC CRL 1593), HL-60 (ATCC CCL 240), and HEL (ATCC TIB 180), were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. William Willer Bar and a service

30 Flow Cytometric Analysis of F1-APC Binding to Cells

Adherent cells were harvested by incubation at 37°C for 5 min in phosphate buffered saline (PBS) containing 0.02% EDTA. Cells were

35 washed twice with EDTA/PBS and then once with Hank's balanced salt solution (HBSS). They were resuspended in HBSS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (binding

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buffer). Cells (1 x 105) were incubated at room temperature for 45 min with FI-APC in the dark. After washing, they were resuspended in the binding buffer containing 0.5 μ g/ml of propidium iodide. Bound Fl-APC was analyzed on a flow cytometer, FACScan (Becton Dickinson). Living cells were gated on a dot plot display of forward-scatter (FSC) versus fluorescence-2 (FL2), and F1-APC binding was detected on the fluorescence-1 (FL-1)

channel. All experiments were performed in 10 duplicate.

125 I-APC binding to HUVEC

Monolayers of HUVEC in 24-well microplates (Costar) (1 x 105 cells per well) were washed twice with EDTA/PBS and once with ice-cold HBSS. Cells were then incubated at 4°C for one hour in the binding buffer with 125 I-APC. After washing three times with ice-cold HBSS, cells were released with the EDTA buffer, and the bound radioactivity was measured in a gamma counter (Isodata 500). To determine non-specific, calcium-independent adsorption of radioactivity, the cells were washed with EDTA/PBS and residual radioactivity in the cell pellet was measured. Non-specific binding of radioactivity was consistently less than 5% of the specific binding. The data was analyzed using the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.).

Construction of HUVEC CDNA Library

Poly-A RNA was isolated from HUVEC (1 x 10 8 cells) using the FastTrack $^{\text{TM}}$ mRNA isolation kit (Invitrogen). cDNA was synthesized from 3 μg of poly-A RNA using a Librarian™ I kit (Invitrogen). A BstX I adaptor was ligated, double stranded cDNA 35 was fractionated by agarose gel electrophoresis, and cDNA longer than 700 bp was ligated into a mammalian expression vector, pEF-BOS (Mizushima and Nagata, 1990; this vector was a kind gift from Dr. S. Nagata). The construct was transfected into E. coli DH1OB by electroporation (Bio-Rad Gene Pulser). The library-consisted of 8 X 106

5 independent colonies with an average size of 2.0 kb.

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Expression Cloning and Sequence Analysis

Approximately 2 x 104 independent colonies

were divided into eight subpools (each containing

2,500 independent colonies) and plasmid DNA was prepared from each subpool. Sub-confluent 293T cells in 24-well microplates were transfected with μg of the DNA by the calcium/phosphate method (Graham and Van Der Eb, (1973) Virology 52, 456-

15 467). After 20 hours, the medium was changed, and culture was continued for another 24 hours. The subpools were screened for F1-APC binding by FACS analysis as described above. The positive library pool was then divided into 20 new pools and

rescreened. After three rounds of screening, 96 individual clones were tested and one positive clone was, identified.

The insert (1.3 kb) was subcloned into pBluescript™ (Stratagene), and the nucleotide

25 sequence was determined using a Sequenase™ version

2.0 DNA Sequencing kit (USB). Nucleotide and protein database search employed the BLAST™ (NCBI) and FASTA™ programs (GCG) with GenBank, EMBL, and SwissProt databases.

30 Northern Blot Analysis

were isolated, electrophoresed through formaldehyde agarose gels and transferred to a nylon membrane (Hybond-N[™], Amersham). The 483 bp Xba I fragment 35° from the 5′ end of the EPCR cDNA was labeled by random priming according to the manufacturer's

instructions (Multiprime DNA labeling system, Amersham) and used for hybridization.

Protein C and APC Binding to HUVEC
Endothelial Cells-in suspension bound

- 5 FL-APC, as monitored by flow cytometry, and demonstrated in Figure 1A. Binding was saturable and Ca²⁺ dependent, as shown by Figure 1B. Optimal binding required at least 1 mM Ca²⁺. FL-APC was displaced from the cell surface by APC and protein
- 16 C'equivalently, as shown by Figure 1C. The homologous Gla-domain containing proteins, protein S, factor X, and its active form, factor Xa, failed to displace bound FI-APC, suggesting that there is a specific binding site for APC on the endothelial
 - cell surface. Protein C binding was dependent on the Gla domain, since recombinant gla-domainless protein C (rGDPC) failed to displace F1-APC.

Detailed binding studies were also performed with 125I-labeTed APC and monolayers of HUVEC, as shown by Figures 2A, 2B, 2C and 2D. The binding analysis indicated 7,000 sites per cell and a Kd=30 nM. This affinity is similar to that estimated from Figure 1.3356

Endothelial cell surface thrombomodulin

can interact with protein C and APC. The Kd

(greater than 1 μM) (Hogg et al., (1992) J. Biol.

Chem. 267, 703-706; Olsen et al., (1992)

Biochemistry 31, 746-754), however, is much higher than that of the binding site described above with respect to the new receptor. Furthermore,

polyclonal and monoclonal antibodies against thrombomodulin that inhibit protein C activation did not inhibit the binding. Protein S also can interact with protein C and APC (Dahlback et al.,

35 (1992) <u>Biochemistry</u> 31, 12769-12777), but F1-APC binding to HUVEC was not influenced by protein S addition. Furthermore, polyclonal and monoclonal

antibodies to protein, S did not inhibit the binding. These results indicate the binding site for protein C and APC on endothelium is distinct from these known molecules.

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Nucleotide and Predicted Protein
Structure Analysis of EPCR

The insert was subcloned into pBluescript, and the nucleotide sequence was determined, as shown in Sequence ID No. 1. The cDNA shown in Sequence ID No. 1 consists of 1302 bp, including a translation initiation ATG codon (AGGATGT, (Kozak, (1986) Cell 44, 283-292) at the 5'-end at nucleotides 25-27 of Sequence ID No. 1. A potential polyadenylation signal sequence, AATAAA, (Proudfoot and Brownlee, (1976) Nature 263, 211-214) begins at nucleotide 1267 of Sequence ID No. 1. just 18 bp upstream of the poly(1) sequence.

No. 1, just 18 bp upstream of the poly(A) sequence.

The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2),

which includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2 is the predicted amino acid sequence of EPCR.

Amino acids 1-15 of Sequence ID No. 2
(MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2
(LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative

transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2.

Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID

No. 2. A potential transmembrane region (Engelman et al., (1986) <u>Annu. Rev. Biophys.. Chem.</u> 15, 321-53) consisting of 23 amino acids was identified at

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the C-terminal end (beginning at amino acid 216 of Sequence ID No. 2).

The protein is predicted to be a type 1 transmembrane protein. The extracellular domain contains four potential N-glycosylation sites and four Cys residues. The cytoplasmic region contains only three amino acids and terminates with a Cys, which could be acylated to something or involved in heterodimer formation with another peptide.

Although described with reference to cloning and expression of the protein encoding sequence, larger amounts of protein can be obtained by expression in suitable recombinant host systems, such as mammalian, yeast, bacteria, or insect cells. Isolation can be facilitated by making antibodies to the recombinant protein which are then immobilized on substrates for use in purification of additional receptors, as described below.

As used herein, the nucleotide sequences encoding the receptor include the sequence shown in Sequence ID No. 1, and sequences having conservative substitutions, additions or deletions thereof which hybridize to Sequence ID No. 1 under stringent conditions. As used herein, the amino acids sequences constituting the receptor include the sequence shown in Sequence ID No. 2, and sequences having conservative substitutions, additions or deletions thereof which form a receptor having functionally equivalent biological activity. It is well known to those skilled in the art what constitutes conservative substitutions, additions or deletions, and which could be readily ascertained as encoding, or forming, a functionally equivalent receptor molecule using the functional assays described herein.

3.7 The hydropathic plot shown in Figures 4a-4cgwas performed according to the method of Goldman et al (Engelman et jal, 1994) (solid line) and that of Kyte and Doolittle (1982) J. Mol. Biol. 157, 105-132 (dotted line) and a second of the record 5 The state of the s revealed that the sequence is related to the centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin (Rothbarth et al., (1993) J. Cell Sci. 104, 19-30), as shown by Figures 5a-5b. The similarity in the published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine EPCR. The sequence of murine EPCR is shown in 15 Figure 6. It is distinct from the published sequence of CCD41. However the second The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily and the murine CD1.2, as also shown by 20 Figures 5a-5b. Based on the homology to CD1/MHC, it is likely that EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d is the most similar to EPCR. In the mouse, CCD41 is associated 25 exclusively with the centrosome during G, but becomes detectable elsewhere during the cell cycle, reaching a maximum during G_2 , except during the G_2/M phase (Rothbarth et al., 1993). EPCR expression appears restricted to endothelium, which would not 30 be expected for a cell cycle associated protein. The identification of the protein C receptor on endothelium suggests that the endothelial cell binds protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind, APC/protein C on negatively charged 35 membrane surfaces that include the endothelium

(Stern et al., (1986) J. Biol. Chem. 261, 713-718),

but this is not cell type specific (Dahlback et al., 1992). Thrombomodulin in complex with thrombin can bind protein EC and APC (Hogg et al., 1992). On endothelium, the protein S binding sites (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745), thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that inflammation can impair protein C pathway function at multiple levels that ends that terms

The homology to the CD1/MHC family of proteins is especially interesting since it provides indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed al, 2 and 3. The extracellular domain of EPCR contains four Cys residues that appear to 15 correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two domains have significant homology to the α l and α 2 domains of the CD1 protein family and the α 2 domain of the MHC class 1 protein, suggesting that these proteins evolved from a common ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites and is rich in & strand structure, suggesting that it may form a ß sheet. Despite the B strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6, 381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR, residues 118-188, contains two 30 additional N glycosylation sites and, like the CD1/MHC family, this domain is predicted to have

Modulation of Inflammation using EPCR.

In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to selectins (Grinnell) (at al., (1994) Glycobiology, 4, (221-226) Modest inhibitory effects of APC have been reported on TNE production (Grey et al., (1993) Transplant Proc. 25, 2913-2914), EPCR

- 5 could contribute to these anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8 (Ledbetter et al., (1985)

 J: Immunol: 134, (4250-4254), it is also possible that the proteins Coreceptor is linked to another
- protein and signal through this second protein.

 One of the CD1 family members, CD1d, has been

 reported to promote Tigell responses, possibly involving binding to CD8 (Panja et al., (1993) J.

 Exp.: Med. 178, 1115-1119) and CD1b has recently been
- reported to serve as an antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind protein C/APC could then be linked either directly or indirectly to signalling via direct interaction with cells of the immune system.
- Since the MHC class of proteins is involved in presentation of proteins to cell receptors, the concept of presentation of protein C/APC to inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved.
- This includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction (Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from unknown substrates.
- To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC was compared to several human cell lines.

 Fl-APC bound strongly only to HUVEC, and not to any of the T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160 nM Fl-APC in the presence of 1.3 mM CaCl₂.

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Binding was analyzed by flow cytometry. Slight binding was demonstrated with the osteosarcoma line, HOS and the epidermold carcinoma cell line, HEp-2. COMPLETE Days, Company of the complete of the complete

Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for w Northern Blot Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and U937. Among the cells lines tested,

10 EPCR mRNA was detected at high levels only in HUVEC. The calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte 15 cell-line U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

Effects of TNF on APC Binding and EPCR MRNA Levels

Several other members of the protein C 20 anticoagulant pathway are subject to regulation by inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin expression and message are known to be reduced by exposure of the cells to TNF (Conway and Rosenberg,

25 1988; Lentz et al., 1991). To determine if a similar process, occurs, with EPCR, HUVEC were treated with TNF and APC binding and expression of EPCR mRNA were examined APC binding to HUVEC decreased in a time dependent fashion. EPCR

activity decreased more rapidly than thrombomodulin antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF- α (10 ng/ml). Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by flow

35 cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

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HUVEC were treated with 10 ng/ml of TNF-α for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message was extracted and detected as described above. results demonstrated that the concentration of EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs at the level of mRNA expression. Enhancement of inflammatory responses by blocking binding of endogenous molecules to ECPCR can be achieved by administration of compounds binding to the receptorato a subject in need of inhibition. The degree of binding is routinely determined using assays such as those described above. Compounds which are effective include antibodies to the protein, fragments of antibodies retaining the binding regions, and peptide fragments of APC which include the Gla region. 20 Inhibition of the inflammatory response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the 25 anticoagulant APC at the surface. Alternatively, the function of EPCR could be enhanced by overexpressing the EPCR in endothelium used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients. 30 The DNA sequence can also be used for screening for other homologous or structurally similar receptor proteins using hybridization AME probes. A season Conservation about the season of the These methods and reagents and 35 pharmaceuticals are more readily understood by reference to the following. Contracted excellences in an about the contract of the second

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Screening of parient samples for

2-20 30 30 30 30 30 entered the the expression of receptor proteins. Patients with Almonbosis or the termination of the hyperinflammatory conditions could be screened for 5 defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using 10 hybridization assays of patient, samples, including blood and tissues - Screening can also be - 1 accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic gulabel, or by isolation of target cells and 15 screening for binding activity as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with 32P. 35S, fluorescein, biotin, 20 or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences. Hybridization Probes (1994) ed. The large rate Reaction conditions for hybridization of 25 an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to consoligonucleotide, depending on factors such as oligonucleotide length mathemnumber of Guand C nucleotides, and the composition of the buffer 30 utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art asconditions approximately 25°C below the melting temperature of a perfectly base-paired doublestranded DNA. Higher specificity is generally ... achieved by employing incubation conditions having

higher temperatures, in other words, more stringent

In general, the longer the sequence

conditions.

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or higher the G and C content, the higher the - temperature and/or salt concentration required. Chapter 11 of the well-known laboratory manual of Sambrook et al., MOLEGULAR CLONING: A LABORATORY MANUAL, 5 second edition, Cold-Spring Harbor Laboratory Press, New York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the 10 factors involved and the level of stringency necessary to quarantee hybridization with ... is specificity and Solver and the contract of The preferred size of a hybridization probe is from 10 nucleotides to 100,000 nucleotides 15 in length. Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, as described in 20 greater detail in the text Molecular Generics, Stent, G.S. and R. Calender, pp. 213-219 (1971). Ideally, the probe should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) lendthemselves to production by automated organic synthetic techniques. Sequences from 100-10,000 **25** nucleotides can be obtained from appropriate restriction endonuclease treatments. The labeling of the smaller probes with the relatively bulky Tenue schemiluminescent moieties may in some cases 30 interfere with the hybridization process. Generation of Antibodies for Diagnostic or Therapeutic Use Antibodies to the receptor proteins can also be generated which are useful in detection, characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of binding.

Antibodies are generated by standard techniques,

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using human or animal receptor proteins. Since the proteins exhibit high evolutionary conservation, it may be advantageous to generate antibodies to a protein of a different species of origin than the 5 species in which the antibodies are to be tested or utilized, looking for those antibodies which are immunoreactive with the most evolutionarily conserved regions. Antibodies are typically generated by immunization of an animal using an 10 adjuvant such as Freund's adjuvant in combination with an immunogenic amount of the protein administered over a period of weeks in two to three week intervals, then isolated from the serum, or used to make hybridomas which express the antibodies in culture. Because the methods for 15 immunizing animals yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or generating less 20 immunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarily-determining hypervariable regions (CDRs) are of non-human origin, whereas all 25 framework regions (FR) of variable domains are products of human genes. These "humanized" antibodies present a lesser xenographic rejection stimulus when introduced to a human recipient. To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method 30 described by Daugherty, et al., (1991) Nucl. Acids Res., 19:2471-2476, incorporated herein by reference, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-

idiotypic ScFv is sequenced by the method of

incorporated herein by reference. Using this

Clackson, T., et al., (1991) Nature, 352:624-688,

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sequence, animal CDRs are distinguished from animal framework regions (ER) based on locations of the CDRs in known sequences of animal variable genes. Kabat, H.A., et al., Sequences of Proteins of 5 : Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA 15 sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further decreased by the use of Pharmacia's (Pharmacia LKB 20 Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short 30 linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigenbinding domain of the antibody. Compared to the intact monoclonal antibody, the recombinant ScFv 35 ...includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof perhase include saline, phosphate buffered saline, and other aqueous

carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in humans (Freund's adjuvant can be used for administration of antibody to animals).

Screening for drugs modifying or altering the extent of receptor function or expression

The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on binding of PC or APC. The in vitro studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved, it is possible to conduct studies in laboratory animals such as mice to predict the effects in humans.

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration in vivo on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991) Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415) can elevate thrombomodulin expression. The ability to

screen such drugs or drugs that block TNF down regulation of EPCR provide an approach to elevating EPCR expression in wive and thus enhancing anticoagulant) and anti-inflammatory activity.

5 Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of inhibition of EPCR function.

Assays for testing compounds for useful 10 activity can be based solely on interaction with the receptor protein, preferably expressed on the surface of transfected cells such as those described above. Proteins in solution or

15 immobilized on inert substrates can also be utilized. These can be used to detect inhibition or enhancement in binding of PC or APC

Alternatively, the assays can be based on interaction with the gene sequence encoding the receptor protein, preferably the regulatory sequences directing expression of the receptor protein. For example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using standard oligonucleotide synthetic chemistry. antisense can be stabilized for pharmaceutical use using standard methodology (encapsulation in a liposome or microsphere; introduction of modified nucleotides that are resistant to degradation or groups which increase resistance to endonucleases, such as phosphorothiodates and methylation), then screened initially for alteration of receptor activity in transfected or naturally occurring

laboratory animals. Typically, the antisense would inhibit expression. However, sequences which block

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cells which express the receptor, then in vivo in

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those sequences which "turn off" synthesis can also be targeted. The synthesis can also

The receptor protein for study can be isolated from either naturally occurring cells or cells which have been genetically engineered to express the receptor, as described in the examples above. In the preferred embodiment, the cells would have been engineered using the intact gene.

Random generation of receptor or receptor encoding sequence binding molecules.

Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" 15 (Szostak, (1992) TIBS 19:89). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 1015 individual sequences in 100 μ g of a 100 nucleotide RNA, to some selection 20 and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 10 to RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

Computer assisted drug design

Computer modeling technology allows

visualization of the three-dimensional atomic

structure of a selected molecule and the rational

design of new compounds that will interact with the

molecule. The three-dimensional construct

typically depends on data from x-ray

crystallographic analyses or NMR imaging of the

selected molecule. The molecular dynamics require

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force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMm and OUANTA programs, Polygen Corporation, Waltham, MA. CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., (1988) Acta Pharmaceutica Fennica 97, 159-166; Ripka, New 25 Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, (1989) Annu. Rev. Pharmacol. Toxiciol. 29, 111-122; Perry and Davies, OSAR: Ouantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, (1989) Proc. R. Soc. Lond. 236, 125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., (1989) J. Am. Chem. Soc. 111, 1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as

BioDesign, Inc., Pasadena, CA., Allelix, Inc,

Mississauga, Ontario, Canada, and Hypercube, Inc.,

BNSDOCID: <WO___9605303A1_I_>

Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Generation of nucleic acid regulators Nucleic acid molecules containing the 5' regulatory sequences of the receptor genes can be 15 used to regulate or inhibit gene expression in vivo. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5, flanking region-gene construct in cells depending on the preference and 20 judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic acid sequences in vivo (see, e.g., Mulligan, (1993) Science, 260, 926-932, United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Recently, a delivery system was developed in which nucleic acid 30 is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al., (1993) Science 261, 209-211; incorporated herein by South Committee of the Control reference). Contraction of September 1981 &

The 5' flanking sequences of the receptor gene can also be used to inhibit the expression of the receptor Fig. For example, an antisense RNA of all or a portion of the 5' flanking region of the , 5 receptor gene can be used to inhibit expression of the receptor in vivo. : Expression vectors (e.g., retroviral expression vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which 10 lis expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286) Accordingly, CDNA containing all or a portion of the sequence of the 5' flanking region of the receptor gene can be inserted into an appropriate expression 15 wector so that supon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the receptor protein gene normally found in the cell? This antisense RNA transcript 20 of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. is of course necessary to select sequences of the 5' flanking region that sare downstream from the transcriptional start sites for the receptor 25 protein gene to ensure that the antisense RNA contains complementary sequences present on the The law of mrna wall and see for those waters a control of a complete of the which is the family Antisense RNA can-be generated in vitro 30 moralso and then inserted into cells. Oligonucleotides can be synthesized on an automated of Milligen-Biosearch, Burlington, MA or ABI Model 380B). In addition, mantisense a contract of 35 deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al.,

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(1978) Proc. Natlu-Acad Ascr. USA 75, 280-284; Zamecnik et al., (1986) Proc. Natl. Acad. Sci., 83, 4143-4146; Wickstrom et al. 20(1988) Proc. Natl. Acad. Scir. USA 85, 1028-1032; Crooke, (1993) FASEB 5 J. 7, 533-539 Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see, se.g.se Offensperger et. al., 10 (1993) EMBO J: 12, e1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothicate oligodeoxynucleotides) ##PCT 'WO#93/01286 Rosenberg et al., (synthesis of sulfurthioated oligonucleotides); Agrawal et al., (1988) Proc. Natl. Acad. Sci. USA 85; 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothicates to inhibit replication of human immunodeficiency virus (1); Sarin et al., (1989) 20 Proc. Natl. Acad. Sci. USA 85, 57448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., (1991) Núcleic Acids Res 19, 747-750 (synthesis of 3 exonuclease-resistant oligonucleotides containing 3 oterminal phosphoroamidate modifications); incorporated 25 herein by reference) is aquene or man, o elem The sequences of the 5' flanking region of receptor protein gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of 30 the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block and the transcription of the general see, e.g., Maher, et al., (1989) Science 245, 725-730; Orson et al., (1991) 35 Nucl. Acids Res. 19, 3435-3441; Postal et al., (1991) Proc. Natl. Acad. Sci. USA 88, 8227-8231;

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Cooney et al., (1988) <u>Science</u> 241, 456-459; Young et al., (1991) Proc. Natl. Acad. Sci. USA 88, _10023-10026; Duval-Valentin et al., (1992) Proc. Natl. Acad. Sci. USA 89, 504-508; Blume et al., 5 (1992) <u>Nucl. Acids Res. 20, 1777-1784; Grigoriev et</u> al., (1992) <u>J. Biol. Chem.</u> 267, 3389-3395. Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides 10 for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989); 15 Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991); Holt et al., (1988) Mol. Cell. Biol. 8, 963-973; Wickstrom et al., (1988) Proc. Natl. Acad. 20 Sci. USA 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson 25 et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); ... 30 again without loss of sequence specificity (Maher American et al., (1989); Grigoriev et al., (1992). Methods to produce or synthesize oligonucleotides are well known in the art. Such 35, methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see Chapters 5, 6) to purely

synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., (1984) Ann. Rev. Biochem. 53, 323-356

(phosphotriester and phosphite-triester methods);
Narang et al., (1980) Methods Enzymol., 65, 610-620
(phosphotriester method). Accordingly, DNA
sequences of the 5 flanking region of the receptor
protein gene described herein can be used to design

and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically within the 5' flanking region of a receptor protein gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to facilitate screening of methods and reagents for manipulation of expression.

Preparation of Receptor Protein Fragments Compounds which are effective for blocking binding of the receptor can also consist 25 of fragments of the receptor proteins, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length receptor protein. These will typically be soluble proteins, i.e., not 30 including the transmembrane and cytoplasmic regions, although smaller portions determined in the assays described above to inhibit or compete for binding to the receptor proteins can also be utilized. It is a routine matter to make appropriate receptor protein fragments, test for binding, and then utilize. The preferred fragm nts are of human origin, in order to minimize potential immunological response. The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. They can also be modified to increase in vivo half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. Based on studies with other peptide

attachment to a carrier molecule or inert substrate. Based on studies with other peptide fragments blocking receptor binding, the IC₅₀, the dose of peptide required to inhibit binding by 50%,

10 ranges from about 1 µM to greater than 10 mM, depending on the peptide size and folding. These ranges are well within the effective concentrations for the *in vivo* administration of peptides, based on comparison with the RGD-containing peptides,

described, for example, in U.S. Patent No.
4,792,525 to Ruoslaghti, et al., used in vivo to
alter cell attachment and phagocytosis. The
peptides can also be conjugated to a carrier
protein such as keyhole limpet hemocyanin by its N-

terminal cysteine by standard procedures such as the commercial Imject kit from Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy.

As noted above, the peptides can be

25 prepared by proteolytic cleavage of the receptor proteins, or, preferably, by synthetic means.

These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, (1964) J. Am. Chem.

30 Soc. 85, 2149, used in U.S. Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other

methods of synthesis are described in U.S. Patent
No. 4,305,872 and 4,316,891. These methods can be
gused to synthesize peptides having identical

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sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a pharmaceutically acceptable acid—or base—addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono—, di—, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives in vivo. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent No. 4,629,784 to Stammer:

The peptides are generally active when administered parenterally in amounts above about 1 μ g/kg of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory disorders, the dosage range will be between 0.1 to 70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered.

Pharmaceutical Compositions

Compounds which alter receptor protein

binding are preferably administered in a

pharmaceutically acceptable vehicle. Suitable

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pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline. For enteral and a series administration, the compound will be incorporated into an inert carrier in tablet, liquid, or capsular form. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature. The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric material (for example, a Pluronic , Assess to consider a color because the Alternatively, the compound may be 15 administered in liposcmes or microspheres (or 15) microparticles) - Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the compound can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patent Nos. 4,906,474, 4,925,673, and

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will any law to a second of Disorders to be treated or a As described berein a variety of compounds can be used to inhibit or enhance. expression of the EPCR The nature of the disorder 5 will determine if the expression should be enhanced or inhibited. For example, based on the studies involving the use of an anti-protein C antibody in combination with cytokine it should be possible to treat solid tumors by enhancing an inflammatory response involving blocking of protein C or 10 activated protein C binding to an cendothelial cell protein C/activated protein C receptor by administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. 15 Similarly, it should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting 20 an inflammatory response involving administration of EPCR or EPCR fragments or substances that upregulate EPCR expression to a patient in need of treatment thereof. In asserbly him in the same on each feares also have birds by the agreed up to the grant trough a 17 vilual eftering him had na haar a 191111 - Professional Configurations will also as the 19 (1835年) 122日 日本日本の大学 1230日 e in a contrakt ook oo kuur da bumma koosanii kumma bi edicara all'illinia di die estalla membre di successi di m និងក្រុងប្រទេស ស្រាស់ សង្ឃាត់ នៅ សត្វសម្រាស់ជីង អង្គរស់នេះស្រាក់សំពី ២០១៦ភ្នំ២៤៦ the contract of the contract o tarbutas i la la contrabación de la moderna de la contrabación de la c and the control of th is a subside of the should make or 100 Million 120 The Control of Participation of the State of .128.35

SEQUENCE LISTING

(i) APPLICANT: Oklahoma Medical Research Foundation (1) GENERAL INFORMATION:

(ii) TITLE OF INVENTION: Cloning and Regulation of an Endothelial Cell Protein

NUMBER OF SEQUENCES: 2 C/Activated Protein C Receptor

CORRESPONDENCE ADDRESS: (iv)

(A) ADDRESSEE: Patrea L. Pabst

(B) STREET: 2800 One Atlantic Center

CITY: Atlanta

STATE: Georgia

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COUNTRY: USA (E)

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COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk (B)

COMPUTER: IBM PC compatible

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OPERATING SYSTEM: PC-DOS/MS-DOS SOFTWARE: Patentin Release #1.0, Version <u>0</u>

* ('Viii) ATTORNEY/AGENT INFORMATION: DE DE LOCTES EOU BELEFERE

REGISTRATION NUMBER: 31,284 (A) NAME: Pabst, Patrea L. <u>m</u>

...

REFERENCE/DOCKET NUMBER: OMRF152 ົບ

TELECOMMUNICATION INFORMATION: (ix)

TELEPHONE: (404) 873-8794 B

(B) . .: .:

(i) SEQUENCE CHARACTERISTICS: INFORMATION FOR SEQ ID NO:1:

(2)

LENGTH: 1302 base pairs

TYPE: nucleic acid

STRANDEDNESS: single TOPOLOGY: linear 9

ANTI-SENSE: NO (<u>†</u> †

FEATURE: (;<u>x</u>

(A) NAME/KEY: misc_feature

GCTTTGCTGA ATTAGTCTGA TAAGTGAATG TTTATCTATC TTTGTGGAAA ACAGATAATG 1020
GAGTIGGGGC AGGAAGCCIA IGCGCCAICC ICCAAAGACA GACAGAAICA CCIGAGGCGI 1080
TCAAAAGATA TAACCAAATA AACAAGTCAT CCACAATCAA AATACAACAT TCAATACTTC 1140
CAGGIGIGIC AGACTIGGGA, IGGGACGCIG ATATAATAGG GTAGAAAGAA GTAACACGAA 1200
GAAGTGGTGG AAATGTAAAA TCCAAGTCAT ATGGCAGTGA TCAATTATTA ATCAATTAAT 1260
AATATTAATA AATTTCTTAT ATTTAAAAAA AAAAAAAA
(2) INFORMATION FOR SEQ ID NO:2: (1) SEQUENCE CHARACTERISTICS: 10 10 10 10 10 10 10 10 10 10 10 10 10
(B) TYPE: amino acid (C) TOPOLOGY: alinears (
(iii) HYPOTHETICAL: NO (ix) FEATURE: A FORT AND A FORT AND A FEATURE: A FORT AND A FORT
E E
(D) OTHER INFORMATION: /note= "Endothelial Cell Protein Receptor encoded by nucleotides 1 through 1302 of Sequence ID No. 1."
22
(B) LOCATION: 1.15 (D) OTHER INFORMATION: /note= "Amino acids 1-15 represent
a putative signal sequence."
(ix) FEATURE: (A) NAME/KEY: Domain
(B) LOCATION: 211236 (D) OTHER INFORMATION: /note= "Amino acids 211-236
(IX) FEALORE: (A) NAME/KEY: Activ -site
(B) TOCATION: 47 174

) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66, 136-138 and 172-174 represent potential N-glycosylation sites."

(ix) FEATURE:

) NAME/KEY: Modified-site

(B) LOCATION: 17.186

(D) OTHER INFORMATION: /note= "Amino acids 17, 114, 118 and 186 represent extracellular cysteine

residues." SEQUENCE DESCRIPTION: SEQ ID NO:2: Phe Leu Thr Thr Leu Leu Pro Ile Leu Leu Leu Ser Gly Trp Ala 5 Met

Gln Cys Ser Gln Asp Ala Ser Asp Gly Leu Gln Arg Leu His Met 13 7 7 2 1 2 9 Cm 1 2 9 Cm

Ala Gln Gly Asn Ser Tyr Phe Arg. Asp. Pro Tyr His Val Trp Tyr

Ile

Pro Asp Leu Gly Gly His Leu Thr His Val Leu Glu Gly 50 ... 55 ... 55 50 **A**la 80 Trp The The Ile Ile Gln Leu Gln Pro Leu Gln Glu Pro Glu Ser

Arg Thr Gln Ser Gly Leu Gln Ser Tyr Leu Leu Gln Phe His Gly Leu 06 85

Pro Leu Thr Leu Ala Phe 105 Val Arg Leu Val His Gln Glu Arg

Arg Cys Phe Leu Gly Cys Glu Leu Pro Pro Glu Gly Ser Arg Ala His 115

Phe Val Ser Phe Arg Ser Ser Asn Gly Phe Glu Val Ala Val 135 Phe Val

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We claim.

- An isolated endothelial cell protein C/activated protein C receptor.
- 2. The receptor of claim 1 encoded by the nucleotide sequence of Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof hybridizing to Sequence ID No. 1 under stringent conditions, which encode the receptor.
- 3. The receptor of claim 1 having the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.
- 4. The receptor of claim 1 expressed on the surface of a non-human cell or a non-endothelial cell.
- 5. The receptor of claim 1 in soluble form.
- 6. The receptor of claim 5 lacking at least a portion of the transmembrane region.
- 7. A nucleotide sequence encoding an endothelial cell protein C/activated protein C receptor.
- 8. The sequence of claim 7 having the nucleotide sequence of Sequence ID No. 1 or degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing under stringent conditions to Sequence ID No. 1.
- 9. The sequence of claim 7 encoding the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.
- 10. The sequence of claim 7 further comprising an expression vector.
- 11. The sequence of claim 10 further comprising an expression host.

A CONTRACTOR OF THE ASSESSMENT OF

- on the surface of a non-human cell or a non-endothelial cell.
- soluble form of the receptor.
- fragment of the receptor of at least fourteen consecutive nucleotides in length.
 - with a detectable label and the sequence of claim 14 labelled
 - inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor comprising administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor.
 - compound is selected from the group consisting of antibodies and fragments of antibodies to the receptor, nucleic acid sequences inhibiting expression of the receptor, and synthetic or natural compounds other than proteins, peptides or nucleic acid sequences which inhibit binding.
 - inflammatory response involving administration of a compound selected from the group consisting of EPCR or EPCR fragments and substances that upregulate EPCR expression to a patient in need of treatment thereof.
 - 19. An antibody or antibody fragment specifically immunoreactive with a unique epitope of an isolated endothelial cell protein C/activated protein C receptor.
 - 20. The antibody of claim 19 wherein the receptor is encoded by the nucleotide sequence of

Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing to Sequence ID No. 1 under stringent conditions.

The antibody of claim 19 wherein the receptor has the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

22. A method for screening for a compound which alters the binding of an endothelial receptor protein to protein C or activated protein C comprising providing an assay for binding of protein C or activated protein C to the receptor protein,

adding the compound to be tested to the way assay, and which was a second to be stested to the

determining if the amount of protein C or activated protein C which is bound to the receptor protein is altered as compared to binding in the absence of the compound to be tested.

23. A method for screening patients for abnormal receptor protein activity or function comprising the presence of an endothelial cell surface receptor binding protein C and activated protein C; and activated protein C; and a comparing the receptor to determine if the quantity present or the function of the receptor is equivalent to that present in normal

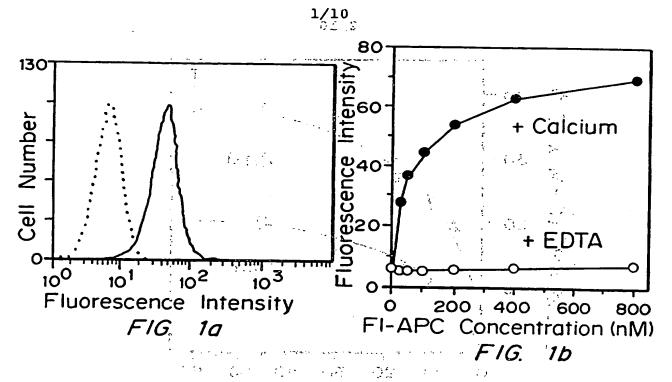
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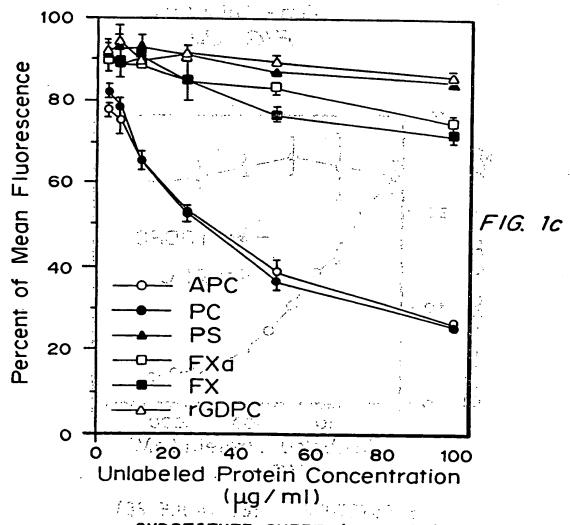
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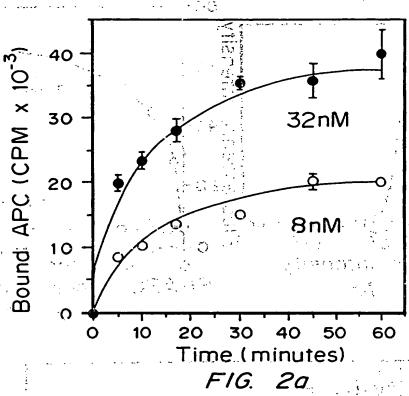
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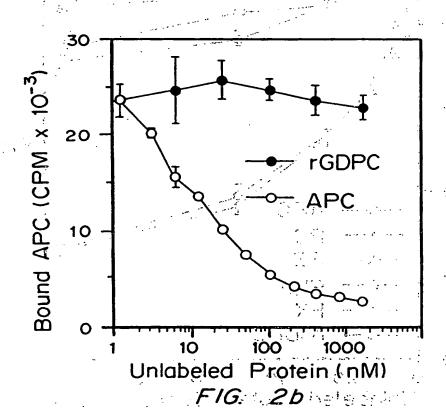
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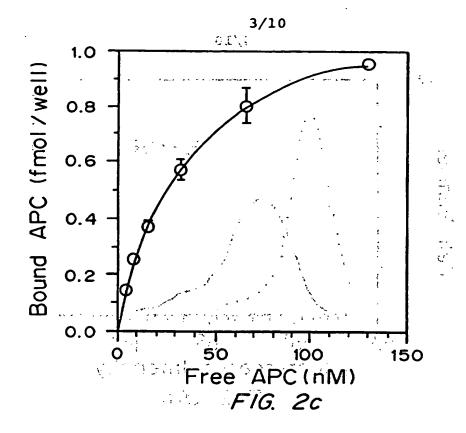


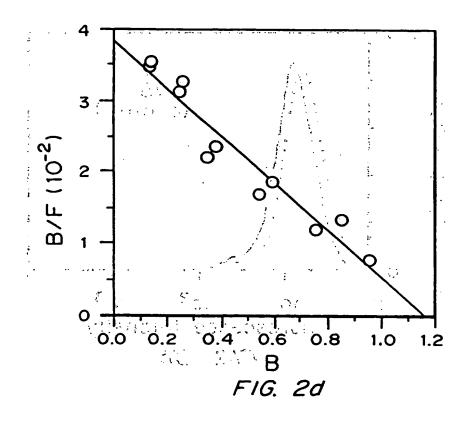




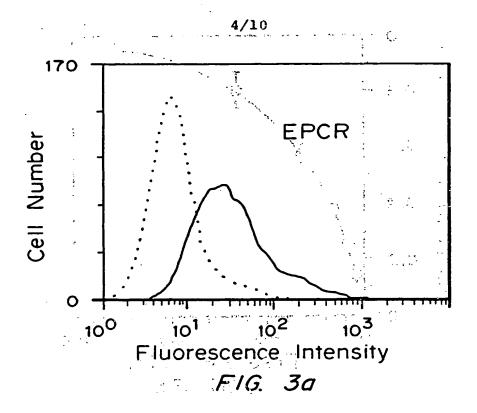


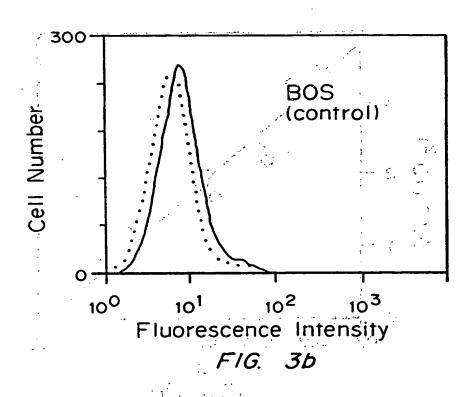
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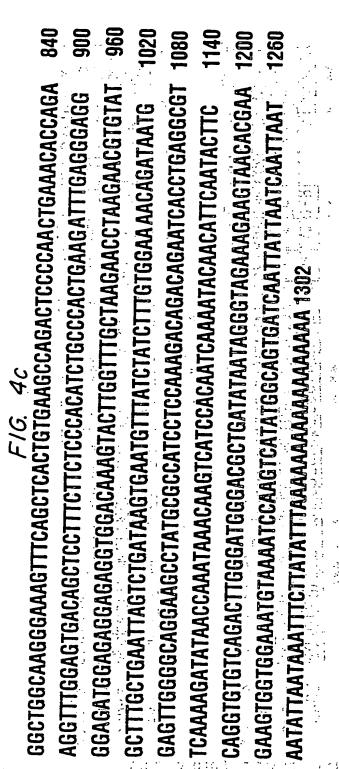
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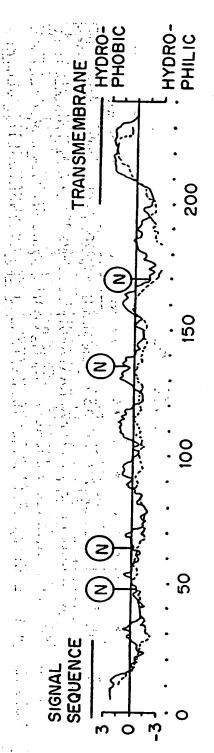
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112 TTCCACGGCCTCGTGCGCCTGGTGCACCAGGAGCGGACCTTGGCCTTTCCTCTGACCATC FHGLVRLVHQERTLAFPLTI

132 CGCTGCTTCCTGGGCTGTGAGCTGCCTCCCGAGGGCTCTAGAGCCCATGTCTTCGAA Ø Œ ш ш — F L G © R (C)

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1 MLTTLLPILLLSGWAFCSQDASDGLQRLH	MLQISYFRDPYHVWYQGNA48
1 MLTKFLPLLLLLPGCALCNSDGSQSLHI	
49 SLGGHLTHVLEGPDTNTTIIQLQPLQEPES\	
99 LVHQERTLAFPLTIRCFLGCELPPEGSF	
144RPERALWQADTQVTSGVVTFTLQQLNAYN	
194 ISAENTKGSQTSRSYTSLVLGVLVGGFIIA .:	

INTERNATIONAL SEARCH 2REPORT

Internal I Application No PCT/US 95/09636

IPC 6	FICATION OF SUBJECT MATTER- C12N15/12 C07K14/705 A61K3 C07K16/28 G01N33/68	9/395 C12N15/11 A61K38/17
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	BALTIMORE, MD, USA, pages 26486-26491, K. FUKUDOME ET AL. 'Identific cloning, and regulation of a neendothelial cell protein C/act protein C receptor.' see the whole document	ation, ovel ivated
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	nt published prior to the international filing date but an the priority date claimed	in the art. *A" document member of the same patent family
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INTERNATIONAL SEARCH REPORT

Interns ul Application No PCT/US 95/09636

CIRCULATION, Confidence to the relevant passages CIRCULATION, Confidence to the relevant passages CIRCULATION, Confidence to the relevant passages 1-3 VOI90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, Cloning, and regulation of a novel'	
vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel	,7-11
vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel	
YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel	
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endothelial cell protein C/activated	
protein C receptor.	
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vol. 72, no. 3, September 1994 STUTTGART,	
vol. 72, no. 3, September 1994 STUTIGART, GERMANY, pages 465-474,	
pages 465-4/4, N. BANGALORE ET AL. 'High affinity	1
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protein C on cultured human umbilical vein	:
endothelial cells.'	
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I THE DUNKING OF DIOCOGIONE CHEFTS IN 1	7,7
vol. 270, no. 10, 10 March 1995 BALTIMORE,	
MD, USA,	- 2
pages 5571-5577, K. FUKUDOME ET AL. 'Molecular cloning and	
expression of murine and bovine	•
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protein C receptor (EPCR).	
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INTERNATIONAL SEARCH REPORT

ntr-national application No

PCT/US 95/ 09636

This international search report has not been established in respect of sertain claims under Article 17(2)(a) for the following reasons: 16-18 Claims Not.	Box I	Observations where certain claims were found unsea	rchable (Continuation of item 1 of first sheet)
1. X Claims Now. 16-18 Secure they relate to subject matter not required to be starched by this Authority, numby. Remark. Although these claims are differeded to a method of treatment of the human/animal body, the Search has been carried out and based on the alleged effects of the compound/composition. 2. Claims Now. Secure they relate to parts of the international application that do not comply with the prescribed requirements to such an extens they need to part of the international earth can be carried out, specifically. 3. Claims Now. Secure they are dependent claims and are not drafted in accordance with the second and third seasoness of Rule 6.4(s). 3ax II Observations where unity of invention is lacking (Continuation of item 2 of first sheet). This International Searching Authority found multiple Inventions in this international application, as follows: As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. As all required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were good, specifically claims Nos.: As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were good, specifically claims Nos.: No present to the invention first mentioned in the claims; it is covered by claims Nos.:	This is		
because they relate to subject maters not required to be searched by this Authority, namely. Remark: Although these claims are differented to a method of treatment of the human/aninal body, the search has been carried out and based on the alleged effects of the compound/composition. 2. Claims Nos: because they relate to parts of the international application that do not comply with the prescribed requirements to such an activity that no meaningful international search can be carried out, specifically: 3. Claims Nos: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a). Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet). This international Searching Authority found multiple inventions in this international application, as follows: As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. As all searchable claims could be searches without effort justifying an admitopal fee, this Authority did not invite payment of any additional fee. As all searchable claims could be searches without effort justifying an admitopal fee, this Authority did not invite payment of any seditional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Noz. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Noz.	וחוצ נחו	ernational search report has not been established in respect	of certain claims under Article 17(2)(a) for the following reasons:
because they relate to subject mater not required to be starched by this Authority, namely. Remark: Although these claims are differented to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition. Claims Nos: because they relate to parts of the international application that do not comply with the prescribed requirements to such an activity that no meaningful international match can be carried out, specifically. Limits Nos: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(s). Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet). This international Searching Authority found multiple inventions in this international application, as follows: As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. As all searchable claims could be exarches without effort justifying an additional search fees, this Authority did not invite payment of any additional fee. As all searchable claims could be exarches without effort justifying an additional fee, this Authority did not invite payment of any sedditional fee. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Not: No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Not: No protest accompanied the payment of additional search fees.	. [V	- 1.1 f. 1	Live in Caratha Contraction Company of the Contraction of the Contract
Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition. 2. Claims Nos: because they relate to parts of the international application that do not comply with the prescribed requirements to such as seans that no meaningful international search can be carried out, specifically: 3. Claims Nos: because they are dependent claims and are not drafted in accordance with the second and third enterests of Rule 6.4(a). 3. Claims Nos: because they are dependent claims and are not drafted in accordance with the second and third enterests of Rule 6.4(a). 3. If the second and third enterests of Rule 6.4(a). 3. As all required additional washed from multiple inventions in this international application, as follows: As all required additional search fees were timely paid by the applicant, this international search report covers all earthable claims. As all required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos: As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos: No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos: The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.	ı. [V	because they relate to subject matter not required to be se	arched by this Authority namely
human/animal body, the search has been carried out and based on the alleged effects of the compound/composition. Claims Nos:		Remark: Although these claims are	directed to a method of treatment of the
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

WO 96/05303 (11) International Publication Number: (51) International Patent Classification 6: C12N 15/12, C07K 14/705, A61K 39/395, 22 February 1996 (22.02.96) (43) International Publicati n Date: C12N 15/11, A61K 38/17, C07K 16/28, t bedosses ed to little in the control of the second to the control of the contro G01N 33/68 (81) Designated States: AU, CA, JP, European patent (AT, BE, PCT/US95/09636 CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, (21) International Application Number: 13000 9 August 1995 (09.08.95) (22) International Filing Date: Published (30) Priority Data: With international search report. 12 August 1994 (12.08.94) (12.08.94) 289,699 Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of (71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDAamendments. TION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US). (72) Inventors: FUKUDOME, Kenji; 13125 West Park Place, Oklahoma City, OK 73142 (US). ESMON, Charles, T., 5800 North Stonewall, Oklahoma City, OK 73111 (US). (74) Agent: PABST, Patrea, L., Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, The control of the same of the control of the contr GA 30309-3450 (US).

(54) Title: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

(57) Abstract

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca²⁺ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

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CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

Background of the Invention

The present invention is generally in the area of cloning, expression, and regulation of an endothelial cell protein C/activated protein C receptor.

Protein C plays a major role in the regulation of blood coagulation. deficient in protein C usually exhibit life threatening thrombotic-complications in infancy (Seligsohn et al., (1984) N. Engl. J. Med. 310, 10 559-562; Esmon, (1992) Trends Cardiovasc. Med. 2, 214-220) that are corrected by protein C administration (Dreyfus et al., (1991) N. Engl. J. Med. 325, 1565-1568). In addition, activated protein C (APC) can prevent the lethal effects of 15 E. coli in baboon models of gram negative sepsis (Taylor et al., (1987) J. Clin. Invest. 79; U.S. Patent No. 5,009,889 to Taylor and Esmon) and preliminary clinical results suggest that protein C is effective in treating certain forms of human 20 septic shock (Gerson et al., (1993) Pediatrics 91, 418-422). These results suggest that protein C may both control coagulation and influence inflammation. Indeed, inhibition of protein S, an 25 important component of the protein C pathway, exacerbates the response of primates to sublethal levels of E. coli and augments the appearance of TNF in the circulation (Taylor et al., (1991) Blood 78, 357-363). The mechanisms involved in controlling the inflammatory response remain 30 unknown.

Protein C is activated when thrombin, the terminal enzyme of the coagulation system, binds to an endothelial cell surface protein, thrombomodulin (Esmon, (1989) J. Biol. Chem. 264, 4743-4746;
Dittman and Majerus, (1990) Blood 75, 329-336;

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Dittman, (1991) Trends Cardiovasc. Med. 1, 331-336). In cell culture, thrombomodulin transcription is blocked by exposure of endothelial cells to tumor necrosis factor (TNF) (Conway and

- Rosenberg, (1988) Mol. Cell. Biol. 8, 5588-5592) and thrombomodulin activity and antigen are subsequently internalized and degraded (Lentz et al., (1991) Blood 77, 543-550, Moore, K.L., et.al., (1989) Blood 73, 159-165). In addition, C4bBP,
- a.regulatory protein of the complement system, binds protein S to form a complex that is functionally inactive in supporting APC anticoagulant activity in vitro (Dahlbäck, (1986) J. Biol. Chem. 261, 12022-12027) and in vivo
 - 15 (Taylor, et al., 1991). Furthermore, C4bBP behaves as an acute phase reactant (Dahlbāck, (1991)

 Thromb. Haemostas. 66, 49-61). Thus, proteins of this pathway not only appear to regulate inflammation, but they also interact with
 - components that regulate inflammation, and they themselves are subject to down regulation by inflammatory mediators.

Given the central role of the protein C pathway in regulating the host response to

- in controlling blood coagulation, it is important to identify and characterize all of the components that interact with the system. This is especially true since the molecular basis of the
- anti-inflammatory effects of the protein C pathway components have yet to be elucidated at the molecular level.

It is therefore an object of the present invention to provide a cellular receptor for protein C and activated protein C.

invention to provide nucleotide sequences encoding

the cellular receptor and amino acid characterization of the receptor which allows expression of recombinant native and modified forms Company of the Committee of the Committee of of the receptor.

It is another object of the present invention to provide methods of modulating the inflammatory response involving protein C and activated protein C.

Summary of the Invention

An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 15 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C -(Kd=30 nM) and is calcium dependent. The message 20 and binding function of the receptor are both down

regulated by cytokines such as TNF.

These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory 25 cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved: Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing 30 EPCR to surfaces in contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of EPCR can be enhanced by 35 overexpressing the EPCR in endothelium that could on the contract of the property of the state of the state

be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

There is the state of the contrasting that they are greatly given. Brief Description of the Drawings Figures 1A, 1B and 1C are flow cytometric 5 analyses of Fl-APC (fluorescent labelled activated protein C) binding to HUVEC (human umbilical vein endothelial cells). Figure 1A is a graph of cell number versus logeof fluorescence intensity, demonstrating F1-APC binding to HUVEC. HUVEC (1 x 10 105) were incubated at room temperature without (dotted line) or with 160 nM of F1-APC (solid line) in the presence of 1.3 mM CaCl2. After washing, bound APC was analyzed by flow cytometry. Figure 18 is a graph of fluorescence intensity versus F1-15 F APC concentration \((nM) \) demonstrating the \(\) concentration dependence of F1-APC binding to mission HUVEC. HUVEC were incubated with F1-APC in the absence (open circles) or presence of 1.3 mM CaCl₂ (closed circles) and binding was measured as in A. Mean channel fluorescence/intensity is plotted for 20 each F1-APC concentrations (between 0 and 800 nM). Figure IC is a graph of the percent of mean fluorescence versus unlabeled protein concentration of the of (µg/ml), demonstrating the effects of unlabeled 25. proteins on F1-APC binding to HUVEC. F1-APC binding to HUVEC was carried out in the presence of mission of the mindicated concentrations (between 0 and 100 - μg/ml) of unlabeled APC, protein C, protein S, v factor X and Xa or recombinant Gla-domainless 1230.0 protein [Ca(rGDPC)], recomposing Supervision () Figures 2Ay 2By, 2C; and 2D are graphs of 125I-APC Binding to HUVEC@Monolayers. ... Figure 2A is entropy of the bound APC (cpm x 10-3) versus time The second (min); showing the time course of 125I-APC binding 9935 % to HUVEC. % HUVEC: monolayers (1.2 x 105 cells) were

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nM (op n squares) 125I-APC. At the indicated times, cells were washed and bound radioactivity was measured. Figure 2B is a graph of bound APC (cpm x 10-3) versus unlabeled protein (nM) demonstrating the effects of unlabeled APC and rGDPC on 125 I-APC binding to HUVEC. HUVEC were incubated at 4°C for one hour with 125 I-labeled APC in the presence of the indicated concentrations (between 01 and approximately 1000 nM) of unlabeled APC (open circles) or rGDPC (closed circles). After washing, bound radioactivity was measured. Figure 2C is a graph of bound APC (fmol/well) versus free APC (nM) demonstrating the concentration dependence of 1251-APC binding to HUVEC . Monolayers of HUVEC were 15 incubated with the concentrations of 1251-APC indicated as described above. Specific binding was determined as described below. Figure 2D is a Scatchard analysis of 2251-APC binding to HUVEC. Each value was calculated from the data shown in 1 20 - Figure 2C. Branco to les brain te per est en co. . Programme of Figures. 3A and 3B are flow cytometric analyses of F1-APC-binding to 293T cells transfected with accDNA clone of EPCR. Cells were transfected with adclone EPCR/pEF-BOS or pEF-BOS 25 (negative control) by the calcium/phosphate method. After 24 h, cells-were harvested and F1-APC binding was performed in the absence (dotted lines) or presence of 1.3 mM CaCl2 (solid lines). e was family Figures 4a-4c areathe predicted protein 30 structure of EPCR based on nucleotide sequence (SEQ ID NO. 1), predicted amino acid sequence (SEQ ID NO. 2) and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated with the solid bars 200 100 100 100 100 Figures 5a-5b are a comparison of the amino acid sequence of EPCR to the amino acid sequences of other members of the CD1 family and CCD41. The EPCR sequence is shown in the first line and

compared to murine CCD41 (second line), human CD1d (third line) and murine CD1.2 (fourth line).

Identities with EPCR are indicated by open boxes.

Residues that are conserved between EPCR and all of the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single asterisk.

Figure 6 is a comparison of the amino
10 acid sequence of human EPCR (first line) to the
amino acid sequence of murine EPCR (second line).
Identities are indicated by lines. Similarities
are indicated with dots.

Detailed Description of the Invention 15 I. Cloning and Characterization of EPCR.

Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably (Kd = 30 nM, 7000 sites per cell) in a Ca²⁺ dependent fashion. FL-APC binding to various human cell lines were examined, and found that the binding was HUVEC specific. A human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was

- constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990)

 Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into
 - 30 293T cells, using the method of Kaisho et al., (1994) Proc. Natl. Acad. Sci. (USA) 91, 5325. FL-APC binding was analyzed on a flow cytometer.

One of eight subpools gave a positive signal. This subpools was divided into 20 subpools and

positive clone, EPCR-1, was isolated. EPCR-1

Programme Carrier Contraction carries a 1.3 kb insert. When transfected into 293T cells. this clone was capable of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

5 Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of this receptor 15 for protein C in regulating the inflammatory englage response. The following many

Materials and Methods

Protein Preparation

Human protein C (Esmon et al., (1993)

- 20 Meths. Enzymol. 222, 359-385), APC (Esmon et al., 1993), recombinant gla domainless protein C (rGDPC) (Rezaie et al., (1992) <u>J. Biol. Chem.</u> 267, 11701-11704), protein S (Taylor et al., 1991), factor X and factor Xa (Le Bonniec et al., (1992)
- 25 J. Biol. Chem. 267, 6970-6976) were prepared as described in the cited publications.

Selective labeling of the active site of APC with fluorescein was performed by the method of Bock (Bock, P.E. (1988) Biochemistry 27,

[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH₂C1, (200 μ M) was reacted with 40 μM APC for 1 hour at room temperature. After dialysis, the covalently modified APC was incubated at room temperature for

35 whome hour with 200 μM 5- (iodoacetamido) fluorescein (Molecular Probes). Free fluorescein was removed by gel filtration on a PD-10 column (Pharmacia).

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... Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical-cords by collagenase treatment and cultured in medium 199 15 % containing 15% fetal bovine serum, 10 μ g/ml \sim which heparin, and 0.5% endothelial cell growth supplement prepared from bovine brain extract (Maciag at al., (1979) Proc. Natl: Acad. Sci. (USA) 76, 5674-5678). HOS (ATCC CRL 1543), HEp-2 (ATCC 20 CCL 23) and 293 cells (ATCC CRL 1573) transformed with SV40 large T antigen (293T, a gift from Dr. Kenji Oritani) were maintained in Earl's MEM supplemented with 10% fetal bovine serum. The human lymphocyte cell lines, Jurkat, MOLT3 (ATCC CRL 1552), Jijoye (ATCC:CCL 87), Raji (ATCC CCL E. 14 1 4 5 86) , EU-937 (ATCC CRL: 1593) , : HL-60 (ATCC CCL 240) , and HEL: (ATCC: TIB 180), were maintained in RPMI-1640 medium supplemented with 10% fetal bovine

Flow Cytometric Analysis of F1-APC Binding to Cells

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Adherent cells were harvested by incubation at 37°C for 5 min in phosphate buffered saline (PBS) containing 0.02% EDTA. Cells were washed twice with EDTA/PBS and then once with Hank's balanced salt solution (HBSS). They were resuspended in HBSS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (binding

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buffer). Cells: (1 x 105) were incubated at room temperature for 45 min with Fl-APC in the dark.

After washing, they were resuspended in the binding buffer containing 0.5 μg/ml of propidium iodide.

- Bound F1-APC was analyzed on a flow cytometer,
 FACScan (Becton Dickinson). Living cells were
 gated on a dot plot display of forward-scatter
 (FSC) versus fluorescence-2 (FL2), and F1-APC
 binding was detected on the fluorescence-1 (FL-1)
 - channel. All experiments were performed in duplicate.

125 I - APC binding to HUVEC

Monolayers of HUVEC in 24-well

microplates (Costar) (10x 105 cells per well) were washed twice with EDTA/PBS and once with ice-cold HBSS. Cells were then incubated at 4°C for one hour in the binding buffer with 125I-APC. After washing three times with ice-cold HBSS, cells were released with the EDTA buffer, and the bound

- radioactivity was measured in a gamma counter

 (Isodata 500). To determine non-specific,
 calcium-independent adsorption of radioactivity,
 the cells were washed with EDTA/PBS and residual
 radioactivity in the cell pellet was measured.
- Non-specific binding of radioactivity was consistently less than 5% of the specific binding.

 The data was analyzed using the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.):

Construction of HUVEC CDNA Library

Poly-A RNA was isolated from HUVEC (1 x 10° cells) using the FastTrack™ mRNA isolation kit (Invitrogen). cDNA was synthesized from 3 μg of poly-A RNA using a Librarian™ I kit (Invitrogen). A BstX I adaptor was ligated, double stranded cDNA was fractionated by agarose gel electrophoresis, and cDNA longer than 700 bp was ligated into a mammalian expression vector, pEF-BOS (Mizushima and

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Nagata, 1990; this vector was a kind gift from Dr. S. Nagata). The construct was transfected into E. coli DH1OB by electroporation (Bio-Rad Gene Pulser^M). The library- consisted of 8 X 10⁶ independent colonies with an average size of 2.0 kb.

Expression Cloning and Sequence Analysis Approximately 2 x 104 independent colonies were divided into eight subpools (each containing 2,500 independent colonies) and plasmid DNA was prepared from each subpool. Sub-confluent 293T cells in 24-well microplates were transfected with 1 μ g of the DNA by the calcium/phosphate method (Graham and Van Der Eb, (1973) Virology 52, 456-467). After 20 hours, the medium was changed, and culture was continued for another 24 hours. The subpools were screened for F1-APC binding by FACS analysis as described above. The positive library pool was then divided into 20 new pools and rescreened. After three rounds of screening, 96 individual clones were tested and one positive clone was identified.

The insert (1.3 kb) was subcloned into pBluescript™ (Stratagene), and the nucleotide sequence was determined using a Sequenase™ version 2.0 DNA Sequencing kit (USB). Nucleotide and protein database search employed the BLAST™ (NCBI) and FASTA™ programs (GCG) with GenBank, EMBL, and SwissProt databases.

Northern Blot Analysis

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Total RNAs (15 μg) from various cells were isolated, electrophoresed through formaldehyde agarose gels and transferred to a nylon membrane (Hybond-NTM, Amersham). The 483 bp Xba I fragment from the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's

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instructions (Multiprime DNA labeling system, Amersham) and used for hybridization.

Protein C and APC Binding to HUVEC Endothelial cells in suspension bound 5 FL-APC, as monitored by flow cytometry, and demonstrated in Figure 1A. Binding was saturable and Ca2 dependent, as shown by Figure 1B. Optimal binding required at least 1 mM Ca2+. FL-APC was displaced from the cell surface by APC and protein C equivalently, as shown by Figure 1C. The homologous Gla-domain containing proteins, protein S, factor X, and its active form, factor Xa, failed to displace bound F1-APC, suggesting that there is a specific binding site for APC on the endothelial cell surface. Protein C binding was dependent on the Gla domain, since recombinant gla-domainless protein C (rGDPC) failed to displace F1-APC.

> Detailed binding studies were also performed with 125 I-labeled APC and monolayers of HUVEC, as shown by Figures 2A, 2B, 2C and 2D. The binding analysis indicated 7,000 sites per cell and a Kd=30 nM. This affinity is similar to that estimated from Figure 1.

Endothelial cell surface thrombomodulin can interact with protein C and APC. The Kd (greater than 1 μ M) (Hogg et al., (1992) <u>J. Biol.</u> Chem. 267, 703-706; Olsen et al., (1992) Biochemistry 31, 746-754), however, is much higher than that of the binding site described above with respect to the new receptor. Furthermore, polyclonal and monoclonal antibodies against thrombomodulin that inhibit protein C activation did not inhibit the binding. Protein S also can interact with protein C and APC (Dahlbäck et al., 35 (1992) <u>Biochemistry</u> 31, 12769-12777), but F1-APC binding to HUVEC was not influenced by protein S addition. Furthermore, polyclonal and monoclonal

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antibodies to protein S did not inhibit th binding. These results indicate the binding site for protein C and APC on endothelium is distinct from these known molecules.

Nucleotide and Predicted Protein Structure Analysis of EPCR

The insert was subcloned into pBluescript, and the nucleotide sequence was determined, as shown in Sequence ID No. 1. The cDNA shown in Sequence ID No. 1 consists of 1302 bp, including a translation initiation ATG codon (AGGATGT, (Kozak, (1986) Cell 44, 283-292) at the 5'-end at nucleotides 25-27 of Sequence ID No. 1. A potential polyadenylation signal sequence, AATAAA, (Proudfoot and Brownlee, (1976) Nature 263,

AATAAA, (Proudfoot and Brownlee, (1976) Nature 263, 211-214) begins at nucleotide 1267 of Sequence ID No. 1, just 18 bp upstream of the poly(A) sequence.

The cDNA is predicted to code for a

protein of 238 amino acids (Sequence ID No. 2),
which includes a 15 amino acid signal sequence (von
Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at
the N-terminal. Therefore, the mature protein is
predicted to contain 223 amino acids. Sequence ID
No. 2 is the predicted amino acid sequence of EPCR.
Amino acids 1-15 of Sequence ID No. 2

(MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2.

Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID

No. 2. A potential transmembrane region (Engelman et al., (1986) <u>Annu. Rev. Biophys.. Chem.</u> 15, 321-53) consisting of 23 amino acids was identified at

the C-terminal end (beginning at amino acid 216 of Sequence ID-No. 2).

transmembrane protein. The extracellular domain contains four potential N-glycosylation sites and four Cys residues. The cytoplasmic region contains only three amino acids and terminates with a Cys, which could be acylated to something or involved in heterodimer formation with another peptide.

Although described with reference to cloning and expression of the protein encoding sequence, larger amounts of protein can be obtained by expression in suitable recombinant host systems, such as mammalian, yeast, bacteria, or insect cells. Isolation can be facilitated by making antibodies to the recombinant protein which are then immobilized on substrates for use in purification of additional receptors, as described below.

As used herein, the nucleotide sequences encoding the receptor include the sequence shown in Sequence ID No. 1, and sequences having conservative substitutions, additions or deletions thereof which hybridize to Sequence ID No. 1 under stringent conditions. As used herein, the amino acids sequences constituting the receptor include the sequence shown in Sequence ID No. 2, and sequences having conservative substitutions, additions or deletions thereof which form a receptor having functionally equivalent biological activity. It is well known to those skilled in the art what constitutes conservative substitutions, additions or deletions, and which could be readily ascertained as encoding, or forming, a functionally equivalent receptor molecule using the functional assays described herein.

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The hydropathic plot shown in Figures 4a-4c was performed according to the method of Goldman et al (Engelman etcal., 1994) (solid line) and that of Kyte and Doolittle (1982) J. Mol. Biol. 157, far is 5500 105-1320 (dotted line) .1800 mag DNA and protein database searches with a received that the sequence is related to the centrosome-associated, cell cycle dependent murin protein, CCD41, also referred as centrocyclin (Rothbarth et al., (1993) J. Cell Sci. 104, 19-30), The similarity in the published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine EPCR : The sequence of murine EPCR is shown in 15 A. Figure 6. A. Italis, distinct from the published sequence of CCD41. The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily and the murine CD1.2, as also shown by 20 Figures 5a-5b. Based on the homology to CD1/MHC, it is likely that EPCR contains two domains consisting of residues 17-114 and 118-188. CD1 family members, CD1d is the most similar to EPCR. In the mouse, CCD41 is associated 25 exclusively with the centrosome during G but to the becomes detectable elsewhere during the cell cycle, reaching a maximum during G_2 , except during the G_2/M - phase (Rothbarth et al., 1993) . EPCR expression appears restricted to endothelium, which would not 30 be expected for a cell cycle associated protein. The identification of the protein C receptor on endothelium suggests that the endothelial cell binds protein C/APC through three distinct mechanisms. In addition to EPCR, protein S.can bind APC/protein Coon negatively charged membrane surfaces that include the endothelium - (Stern et al., (1986) J. Biol. Chem. 261, 713-718),

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(1,2,1) (2,2,3) in expect of eq. () the graph (3,2,3)but this is not cell-type specific (Dahlbäck et al., 1992). Thrombomodulingin complex with thrombin can bind protein Coand APC (Hogg et al., 1992). On endothelium, the protein S binding sites 5 (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745), thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that inflammation can impair protein C pathway function at multiple_levels.to at a multiple_levels.to The homology to the CD1/MHC family of proteins is especially interesting since it provides indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed α 1,2 and 3.00 The extracellular domain of EPCR $_{\odot}$ contains four Cys residues that appear to correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two domains have significant homology to the α l and α 2 domains of the CD1 protein family and the α2 domain 20 of the MHC class 1 protein, suggesting that these proteins evolved from a common ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites and is rich in & strand structure, suggesting that it may form a ß 25 sheet. Despite the Batrand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6, 381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR, residues 118-188, contains two 30 additional N glycosylation sites and, like the CD1/MHC family, this domain is predicted to have limited & structure. Modulation of Inflammation using EPCR.

II. In vitro studies have suggested 35 anti-inflammatory activities for APC. For instance, an unusual carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to

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selectins (Grinnell, at. al., (1994) Glycobiology, 4, 221-226) Modest inhibitory effects of APC have been reported on TNF production (Grey et al., (1993) <u>Transplant. Proc.</u> 25, 2913-2914). PEPCR 5 could contribute to these anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8 (Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the proteins C receptor is linked to another protein and signal, through this second protein. One of the CD1 family members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8 (Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been 15 reported to serve as an antigen presenting molecule (Porcelli et al., (1992) <u>Nature</u> 360, 593-597). ability to bind protein C/APC could then be linked either directly or indirectly to signalling via direct interaction with cells of the immune system. 20 Since the MHC class of proteins is involved in presentation of proteins to cell receptors; the concept of presentation of protein C/APC to inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. 25 This includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction (Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from unknown substrates. 20 30 miles of the BPCR mRNA Levels and APC Binding To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC was compared to several human cell lines.

Fir-APC bound strongly only to HUVEC, and not to any of the T. B. or monocytic cell lines tested. Cells were incubated at room temperature without or with

160 nM F1 APC in the presence of 1.3 mM CaCl2.

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Binding was analyzed by flow cytometry. Slight binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line, HEp-2: ES-ETHS LATE CHASE COMMON ACCOUNTS

5 Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and U937. Among the cells lines tested,

10 EPCR mRNA was detected at high levels only in HUVEC. The calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. prolonged exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte cell line U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

Effects of TNF on APC Binding and EPCR

Several, other members of the protein C ្នាស់ ស្នាស់ ស្នាស់ ស្នាស់ និសាស 20. anticoagulant pathway are subject to regulation by inflammatory cytokines (Esmon, 1989). instance, endothelial cell surface thrombomodulin expression and message are known to be reduced by exposure of the cells to TNF (Conway and Rosenberg,

25 - 1988; Lentzet al. (1991) To determine if a dis similar process occurs with EPCR, HUVEC were treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to EUVEC decreased in a time dependent fashion. EPCR

activity decreased more rapidly than thrombomodulin antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF- α (10 ng/ml). Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by flow

35, cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

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HUVEC were treated with 10 ng/ml of TNF-α for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message was extracted and detected as described above. The results demonstrated that the concentration of EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs at the level of mRNA expression.

- blocking binding of endogenous molecules to ECPCR can be achieved by administration of compounds binding to the receptor to a subject in need of inhibition. The degree of binding is routinely determined using assays such as those described above. Compounds which are effective include antibodies to the protein, fragments of antibodies retaining the binding regions, and peptide fragments of APC which include the Gla region.
 - Inhibition of the inflammatory response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be enhanced by overexpressing the EPCR in endothelium used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.
- The DNA sequence can also be used for screening for other homologous or structurally similar receptor proteins using hybridization probes.
- These methods and reagents and

 35 pharmaceuticals are more readily understood by reference to the following.

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Screening of patient samples for expression of receptor proteins.

Patients with thrombosis or
hyperinflammatory conditions could be screened for
defects in the EPCR gene. Sequence ID No. 1, and
consecutive portions thereof of at least about

- seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic
- label, or by isolation of target cells and

 screening for binding activity, as described in the
 examples above. Typically, one would screen for
 expression on either a qualitative or quantitative
 basis, and for expression of functional receptor.
 Labelling can be with 32P, 35S, fluorescein, biotin,
 - 20 or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

Hybridization Probes

Reaction conditions for hybridization of

- an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer
 - 30 utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-
 - stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words, more stringent conditions. In general, the longer the sequence

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or higher the G and C content, the higher the temperature and/or salt concentration required. Chapter 11 of the well-known laboratory manual of Sambrook et al., Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, New, York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

The preferred size of a hybridization probe is from 10 nucleotides to 100,000 nucleotides 15 in length. Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, as described in 20 greater detail in the text Molecular Genetics, Stent, G.S. and R. Calender, pp. 213-219 (1971). the probe should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) lend themselves to production by automated organic 25 synthetic techniques. Sequences from 100-10,000 nucleotides can be obtained from appropriate restriction endonuclease treatments. The labeling of the smaller probes with the relatively bulky chemiluminescent moieties may in some cases . 30 interfere with the hybridization process.

Generation of Antibodies for Diagnostic or Therapeutic Use

Antibodies to the receptor proteins can also be generated which are useful in detection,

35 characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of binding.

Antibodies are generated by standard techniques,

using human or animal preceptor proteins. Since the proteins exhibit high evolutionary conservation, it may be advantageous to generate antibodies to a protein of a different species of origin than the 5 species in which the antibodies are to be tested or utilized, looking for those antibodies which are immunoreactive with the most evolutionarily conserved regions. Antibodies are typically generated by immunization of an animal using an 10 adjuvant such as Freund's adjuvant in combination with an immunogenic amount of the protein administered over a period of weeks in two to three week intervals, then isolated from the serum, or used to make hybridomas which express the 15 antibodies in culture. Because the methods for immunizing animals yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or generating less 20 minmunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarily-determining hypervariable regions (CDRs) are of non-human origin, whereas all 1250 framework regions (FR) of variable domains are products of human genes. These "humanized" antibodies present a lesser xenographic rejection stimulus when introduced to a human recipient. To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method described by Daugherty, et al., (1991) Nucl. Acids Res., 19:2471-2476, incorporated herein by reference, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-35 idiotypic ScFv is sequenced by the method of Clackson, T., et al., (1991) Nature, 352:624-688, incorporated herein by reference. Using this

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sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. ... Kabat, H.A., et al., Sequences of Proteins of 5 Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further decreased by the use of Pharmacia's (Pharmacia LKB 20 Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes 25 are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short 30 linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigenbinding domain of the antibody. Compared to the intact monoclonal antibody, the recombinant ScFv 35 includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof. These include saline, phosphate buffered saline, and other aqueous 5 carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in 10 humans (Freund's adjuvant can be used for administration of antibody to animals):

Screening for drugs modifying or altering the extent of receptor function or expression

The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on binding of PC or APC. in vitro studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved, it is possible to conduct studies in laboratory animals such as mice to predict the effects in

> In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration in vivo on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991) Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415) can elevate thrombomodulin expression. The ability to

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screen such drugs or drugs that block TNF down regulation of EPCR provide an approach to elevating EPCR expression in vivo and thus enhancing anticoagulant and anti-inflammatory activity.

- Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of inhibition of EPCR function.
- Assays for testing compounds for useful activity can be based solely on interaction with the receptor protein, preferably expressed on the surface of transfected cells such as those described above. Proteins in solution or immobilized on inert substrates can also be utilized. These can be used to detect inhibition or enhancement in binding of PC or APC

Alternatively, the assays can be based on interaction with the gene sequence encoding the receptor protein, preferably the regulatory 20 sequences directing expression of the receptor protein. For example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using standard oligonucleotide synthetic chemistry. The 25 antisense can be stabilized for pharmaceutical use using standard methodology (encapsulation in a liposome or microsphere; introduction of modified nucleotides that are resistant to degradation or groups which increase resistance to endonucleases, such as phosphorothiodates and methylation), then screened initially for alteration of receptor activity in transfected or naturally occurring cells which express the receptor, then in vivo in laboratory animals. Typically, the antisense would 35 inhibit expression. However, sequences which block

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those sequences which "turn off" synthesis can also be targeted.

The receptor protein for study can be isolated from either naturally occurring cells or cells which have been genetically engineered to express the receptor, as described in the examples above. In the preferred embodiment, the cells would have been engineered using the intact gene.

Random generation of receptor or receptor encoding sequence binding molecules.

Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" (Szostak, (1992) TIBS 19:89). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 1015 individual sequences in 100 μ g of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 1010 RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

Computer assisted drug design
Computer modeling technology allows
visualization of the three-dimensional atomic
structure of a selected molecule and the rational
design of new compounds that will interact with the
molecule. The three-dimensional construct
typically depends on data from x-ray
crystallographic analyses or NMR imaging of the
selected molecule. The molecular dynamics require

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1. 2 force field data on The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. 5 Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually 10 coupled with user-friendly, menu-driven interfaces between the molecular design program and the user. Examples of molecular modelling systems are the CHARMm and QUANTA programs, Polygen Corporation, Waltham, MA. CHARMm performs the 15 energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the 20 behavior of molecules with each other. A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., (1988) Acta Pharmaceutica Fennica 97, 159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and 25 Rossmann, (1989) Annu. Rev. Pharmacol. Toxiciol. 29, 111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 30 (1989) Proc. R. Soc. Lond. 236, 125-140 and 141ma with respect to a model receptor for nucleic acid components, Askew, et al., (1989) J. Am. -Chem. -Soc. :111; 1082+1090. Other computer programs that screen and graphically depict 35 chemicals are available from companies such as BioDesign, Inc., Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube, Inc.,

Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Generation of nucleic acid regulators Nucleic acid molecules containing the 5' -regulatory sequences of the receptor genes can be used to regulate or inhibit gene expression in vivo. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene construct in cells depending on the preference and ar judgment of the skilled practitioner (see, e.g., Sambrook et ala, Chapter 16). Furthermore, a númber of viral and Hénviral vectors are being developed that enable the introduction of nucleic 25 acid sequences in vivo (see, e.g., Mulligan, (1993) Science 2607 9264932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference) . Recently, a delivery system was developed in which nucleic acid

is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al.,

35 (1993) Science 261, 209-211; incorporated herein by reference).

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The 5'-flanking sequences of the receptor gene can also be used to inhibit the expression of the receptor For example, an antisense RNA of all or a portion of the 5% flanking region of the 5. receptor gene can be used to inhibit expression of the receptor in vivo. Expression vectors (e.g., retroviral expression vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which 10 is expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286). Accordingly, DNA containing all or a portion of the sequence of the 5' flanking region of the receptor gene can be inserted into an appropriate expression 15 vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the receptor protein gene normally found in the cell. This antisense RNA transcript 20 of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. is of course necessary to select sequences of the 5' flanking region that are downstream from the 25 transcriptional start sites for the receptor protein gene to ensure that the antisense RNA contains complementary sequences present on the one to make the source of the source of the source of . The standard of Antisense RNA can be generated in vitro 30 also, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 3,80B). In addition, antisense 35 deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and

viral replication (see e.g., Zamecnik et al.,

(1978) <u>Proc. Natl. Acad Sci. USA</u> 75, 280-284; Zamecnik et al (1986) Proc Natl. Acad. Sci., 83, 4143-4146; Wickstrom et alpo, (1988) Proc. Natl. Acad. Sci. USA 85, 1028-1032; Crooke, (1993) FASEB 5 J. 7, 533-539. Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see, e.g., Offensperger et. al., 10 (1993) EMBO J. 12, 1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothicate oligodeoxynucleotides); PCT WO-93/01286 Rosenberg et al., (synthesis of sulfurthioate oligonucleotides); Agrawal et al., (1988) Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothicates to inhibit replication of human immunodeficiency virus-1); Sarin et al., (1989) 20 Proc. Natl. Acad. Scill USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., (1991) <u>Nucleic Acids Res</u> 19, 747-750 (synthesis of 3 exonuclease-resistant oligonucleotides containing 30 terminal phosphoroamidate modifications); incorporated Therein by reference) to same the control date of The sequences of the 50 flanking region of receptor protein gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., Maher et al., (1989) Science 245, 725-730; Orson et al:, (1991) 35 Nucl. Acids Res. 19, 3435-3441; Postal et al., (1991) Proc. Natl. Acad. Sci. USA 88, 8227-8231;

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Cooney et al., (1988) Science 241, 456-459; Young et al., (1991) Proc. Natl. Acad. Sci. USA 88, 10023-10026; Duval-Valentin et al., (1992) Proc. Natl. Acad. Sci. USA 89, 504-508; Blume et al., 5 (1992) <u>Nucl. Acids Res.</u> 20, 1777-1784; Grigoriev et al., (1992) J. Biol. Chem. 267, 3389-3395. Fig. 11. Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides 10 for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For a ample, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989); 15 Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991); Holt et al., (1988) Mol. Cell. Biol. 8, 963-973; Wickstrom et al., (1988) Proc. Natl. Acad. 20 Sci. USA 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson 25 et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); 30 again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992). all calls when you Methods to produce or synthesize oligonucleotides are well known in the art. 35 methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely

synthetic-methods, for example, by the cyanoethyl phosphoramidite method asing a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., (1984) Ann. Rev. Biochem. 53, 323-356 5 (phosphotriester and phosphite-triester methods); Narang et al., (1980) Methods Enzymol., 65, 610-620 (phosphotriester method). Accordingly, DNA sequences of the 5' flanking region of the receptor protein gene described herein can be used to design 10 and construct cligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically . 15 within the 5' flanking region of a receptor protein gene in order to inhibit expression of the gene. In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to 20 facilitate screening of methods and reagents for manipulation of expression. Preparation of Receptor Protein Fragments Compounds which are effective for blocking binding of the receptor can also consist 25 of fragments of the receptor proteins, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length receptor protein. will typically be soluble proteins, i.e., not 30 including the transmembrane and cytoplasmic regions, although smaller portions determined in the assays described above to inhibit or compete for binding to the receptor proteins can also be utilized. It is a routine matter to make 35 appropriate receptor protein fragments, test for binding, and then utilize. The preferred fragments are of human origin, in order to minimize potential

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immunological response . The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. can also be modified to increase in vivo half-life, . 5 ... by chemical modification of the amino acids or by attachment to a carrier molecule or inert esubstrate. Based on studies with other peptide fragments blocking receptor binding, the IC_{50} , the dose of peptide required to inhibit binding by 50%, ranges from about 1 μM to greater than 10 mM, depending on the peptide size and folding. These ...ranges are well within the effective concentrations for the ingvivo administration of peptides, based on comparison with the RGD-containing peptides, 15 g. described, for example, in U.S. Patent No. 4,792,525 to Ruoslaghti, et al., used in vivo to alter cell attachment and phagocytosis. The peptides can also be conjugated to a carrier protein such as keyhole limpet, hemocyanin by its N-20 terminal cysteine by standard procedures such as the commercial Imject kit from Pierce Chemicals or expressed as a fusion protein, which may have The dincreased efficacy. The grant when you are the control of the As noted above, the peptides can be prepared by proteolytic cleavage of the receptor 25 proteins, or, preferably, by synthetic means. These methods are known to those skilled in the An example is the solid phase synthesis described by J. Merrifield, (1964) J. Am. Chem. 30 % Soc. 85, 2149, used in U.S. Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other 35 % methods of synthesis are described in U.S. Patent No. 24, 305, 872; and 4, 316, 891; These methods can be used to synthesize peptides having identical

sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as

formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide,

potassium hydroxide, and organic bases such as mono, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives in vivo. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent

No. 4,629,784 to Stammer.

The peptides are generally active when administered parenterally in amounts above about 1 μg/kg of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory disorders, the dosage range will be between 0.1 to

70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered.

Pharmaceutical Compositions

Compounds which alterareceptor protein binding are preferably administered in a pharmaceutically acceptable vehicle. Suitable

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pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline. For enteral administration, the compound will be incorporated into an inert carrier in tablet, liquid, or capsular form. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature.

The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric material (for example, a Pluronic^m,

g and BASF). There was arrest mag to be grown than the Alternatively, the compound may be 15 administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating 20 biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is by G. Gregoriadis, 25 Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal 30 tract directly into the bloodstream. Alternatively, the compound can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, 35 ranging from days to months. See, for example, U.S. Patent Nos. 4,906,474, 4,925,673, and 3,625,214.

Disorders to be treated ంగు ఓైట్లా ఉంటుతున్ని లోని మనిగును క and the As described herein, a variety of compounds can be used to inhibit or enhance expression of the EPCR. The nature of the disorder 5 will determine if the expression should be enhanced or inhibited. For example, based on the studies involving the use of an anti-protein C antibody in combination with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory 10 response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor by administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting 20 an inflammatory response involving administration of EPCR or EPCR fragments or substances that upregulate EPCR expression to a patient in need of treatment thereof: " stage lower than the The progress of the contract was the fixed the factor that in a figure of the control of the second control of the second of Burghout the continue to supply the most quality of the equation ಕ್ಷಾಪ್ರ ಮುಂಚು ಕಷ್ಟ್ರಿಯ ಕ್ಷಣ್ಣ ಕರ್ಮದ ಪ್ರಾಥಾಗಿ ಮುಖ್ಯಾಗಿ ಮಾಡುವ ಮಾಡುವುದ ಬರಿಕೆ ಕ e procedular com a recent operation and the section of the recent Eller and the first trade contains their algebraiches bearing and being their Control of the second second of the first of the second where it that is the control of the en le egit la companya de la company and the second of the second of the second of the second THE COURT OF THE PARTY OF THE P the company of the terms of the part of the part.

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SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT: Oklahoma Medical Research Foundation

TITLE OF INVENTION: Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor (ii)

(111) NUMBER OF SEQUENCES: 2.

(iv)

(A) ADDRESSEE: Patrea L. Pabst. CORRESPONDENCE ADDRESS:

200 1201 West Peachtree Street (B) STREET: 2800 One Atlantic Center

CITY: Atlanta 9

STATE: Georgia

COUNTRY: USA

(E)

ZIP: 30309-3450 (A)

000 000 W COMPUTER READABLE FORM: 3

3.0115 Oct 5

MEDIUM TYPE: Floppy disk B (B)

COMPUTER: IBM PC compatible

(viii) ATTORNEY/AGENT INFORMATION: PROPERTY OF THE PROPERTY OF OPERATING SYSTEM: PC-DOS/MS-DOS SOFTWARE: Patentin Release #1.0, Version #1.25 â

REGISTRATION NUMBER: 31,284 NAME: Pabst, Patrea L. (B)

REFERENCE/DOCKET NUMBER: OMRF152 ີບ

. . . .

TELECOMMUNICATION INFORMATION: TELEPHONE: (404) 873-8794 B (ix)

TELEFAX: (404) 873-8795 (B)

(i) SEQUENCE CHARACTERISTICS: (2) INFORMATION FOR SEQ ID NO:1:

LENGTH: 1302 base pairs TYPE: nucleic acid E (B)

STRANDEDNESS: single TOPOLOGY: linear 9 ΰ

MOLECULE TYPE: CDNA (iii)

ANTI-SENSE: NO (iv)

FEATURE: (ix)

(A) NAME/KEY: misc feature

	a)													•			
	the												•				
	encode	9	120	180	240	300	360	420	480	540	009	099	720	780	840	900	096
	(B) LOCATION: 1.1302 (D) OTHER INFORMATION: /note= "Nucleotides 25 through 738 encode Endothelial Cell Protein Receptor of Sequence ID No. 2." (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:	r gergererer			·												GGAGATGGAG AGGAGAGGTG GACAAAGTAC TTGGTTTGCT AAGAACCTAA GAACGTGTAT
•	Endot)	CAGGT	GGCTG	ATCTC	CACCT	TTGCA	TTCCA	CGCTG	GTGGC	GACAC	CGCAC	CATAI	CTGGC	ACAGO	GGCT	AGGT	GGAG

GCTTTGCTGA ATTAGTCTGA TAAGTGAATG TTTATCTATC TTTGTGGAAA ACAGATAATG	1020
GAGTTGGGGC AGGAAGCCTA TGCGCCATCC TCCAAAGACA GACAGAATCA CCTGAGGCGT	1080
TCAAAAGATA TAACCAAATA AACAAGTCAT CCACAATCAA AATACAACAT TCAATACTTC	1140
CAGGTGTGTC AGACTTGGGA TGGGACGCTG ATATAATAGG GTAGAAAGAA GTAACACGAA	1200
GAAGTGGTGG AAATGTAAAA TCCAAGTCAT ATGGCAGTGA TCAATTATTA ATCAATTAAT	1260
AATATTAATA AATTTCTTAT ATTTAAAAA AAAAAAAA	1302
(2) INFORMATION FOR SEQ ID NO.2:	
(1) SECUENCE CHARACTERISTICS: (A) LENGTH: 238 amino acids	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: protein (iii) HYPOTHETICAL: NO	
FEAT	
(A) NAME/KEY: misc feature (B) LOCATION: 1 355	
<u>a</u> '	entor encoded by
nucleotides 1 through 1302 of Sequence ID No. 1." (ix) FEATURE:	great encourage by
(A) NAME/KEY: Modified-site	
OTHER	
(ix) FEATHER.	
(A) NAME/KEY: Domain	
(B) LOCATION: 211.236	
represent a putative transmember 11-236	
URE:	
(A) NAME/KEY: Activ -site (B) LOCATION: 47, 174	

OTHER INFORMATION: /note= "Amino acids 47-49, 64-66 136-138 and 172-174 represent potential N-glycosylation sites." <u>e</u>

FEATURE: (ix)

NAME/KEY: Modified-site

LOCATION: 17..186

OTHER INFORMATION: /note= "Amino acids 17, 114 and 186 represent extracellular cysteine

SEQUENCE DESCRIPTION: SEQ ID NO:2:

(xi;)

Met Leu Thr Ihr Leu Leu Pro Ile Leu Leu Leu Ser Gly Trp Ala Phe

Cys ser Gln Asp Ala Ser Asp Gly Leu Gln Arg Leu His Met Leu Gln 1.20 1.1. Bant 6 ad

Ile Ser Tyr Phe Arg Asp, Pro Tyr His Val Trp Tyr Gln Gly Asn Ala

Ser Leu Gly Gly His Leu Thr His Val Leu Glu Gly Pro Asp Thr Asn

Thr Thr Ile Ile Gln Leu Gln Pro Leu Gln Glu Pro Glu Ser Trp Ala

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Arg Thr Gln Ser Gly Leu Gln Ser Tyr Leu Leu Gln Phe His Gly Leu

Val Arg Leu Val His Gln Glu Arg Thr Leu Ala Phe Pro Leu Thr Ile 105 100

Arg Cys Phe Leu Gly Cys Glu Leu Pro Pro Glu Gly Ser Arg Ala His

Phe Phe Glu Val Ala Val Asn Gly Ser Ser Phe Val Ser Phe Arg Val

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The first of the f CONTRACTOR SERVICE Gln dla E Gly G1y 235 Ala 170 ÇyB Gly Asp Ala Ten particular of the Color of Gln 10.10 0.00 20 1.22 1.03 10.00 0.00 20 1.22 1.03 Gly 1 Leu Arg Phe Thr Val

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ନ୍ତିକ୍ରିକ ବର୍ଷ ପ୍ରଥମ ଅଧିକ ହେଉଛି । ଅଧିକ ବର୍ଷ ବର୍ଷ । ଏହା ଅଧ୍ୟକ୍ତି ଓଡ଼ିଆ ଅଧିକଥିଲା ଅଧିକ ବର୍ଷ ଓଡ଼ିଆ କଥିଲା । ଜୁଣ୍ଡ ଓଡ଼ିଆ

We claim.

- An isolated endothelial cell protein
 C/activated protein C receptor.
- 2. The receptor of claim 1 encoded by the nucleotide sequence of Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof hybridizing to Sequence ID No. 1 under stringent conditions, which encode the receptor.
 - 3. The receptor of claim 1 having the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.
 - 4. The receptor of claim 1 expressed on the surface of a non-human cell or a non-endothelial cell.
 - 5. The receptor of claim 1 in soluble form.
 - 6. The receptor of claim 5 lacking at least a portion of the transmembrane region.
 - 7. A nucleotide sequence encoding an endothelial cell protein C/activated protein C receptor.
 - 8. The sequence of claim 7 having the nucleotide sequence of Sequence ID No. 1 or degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing under stringent conditions to Sequence ID No. 1.
 - 9. The sequence of claim 7 encoding the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.
 - 10. The sequence of claim 7 further comprising an expression vector.
 - 11. The sequence of claim 10 further comprising an expression host.

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- on the surface of varinon-human cellion a non-endothelial cell.
 - 13. The sequence of claim 7 encoding a soluble form of the receptor.
- fragment of the receptor of at least fourteen consecutive nucleotides in length.
 - 15. The sequence of claim 14 labelled with a detectable label.
- inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor comprising administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor.
- 17. The method of claim 16 wherein the compound is selected from the group consisting of antibodies and fragments of antibodies to the receptor, nucleic acid sequences inhibiting expression of the receptor, and synthetic or natural compounds other than proteins, peptides or nucleic acid sequences which inhibit binding.
 - inflammatory response involving administration of a compound selected from the group consisting of EPCR or EPCR fragments and substances that upregulate EPCR expression to a patient in need of treatment thereof.
 - 19. An antibody or antibody fragment specifically immunoreactive with a unique epitope of an isolated endothelial cell protein C/activated protein C receptor.
 - 20. The antibody of claim 19 wherein the receptor is encoded by the nucleotide sequence of

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Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing to Sequence ID No. 1 under stringent conditions.

21. The antibody of claim 19 wherein the receptor has the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

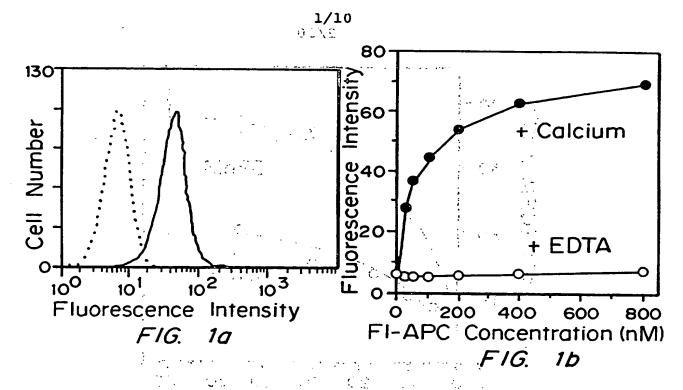
compound which alters the binding of an endothelial receptor protein to protein C or activated protein C comprising providing an assay for binding of protein C or activated protein C to the receptor protein, adding the compound to be tested to the

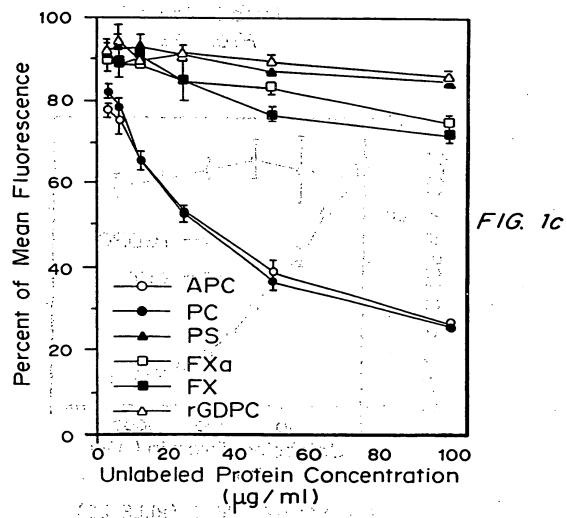
determining if the amount of protein C or activated protein C which is bound to the receptor protein is altered as compared to binding in the absence of the compound to be tested.

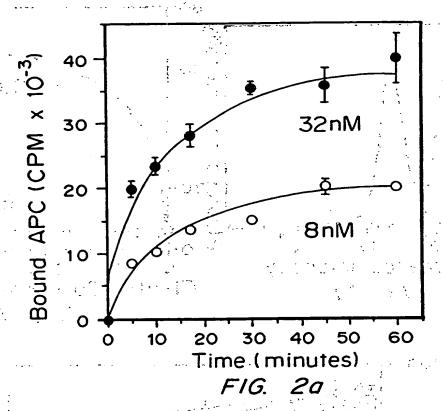
23. A method for screening patients for abnormal receptor protein activity or function

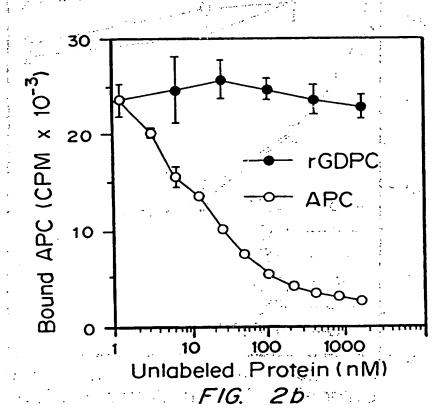
comprising determining the presence of an endothelial cell surface receptor binding protein C and activated protein C, and comparing the receptor to determine if the quantity present or the function of the receptor is equivalent to that present in normal cells.

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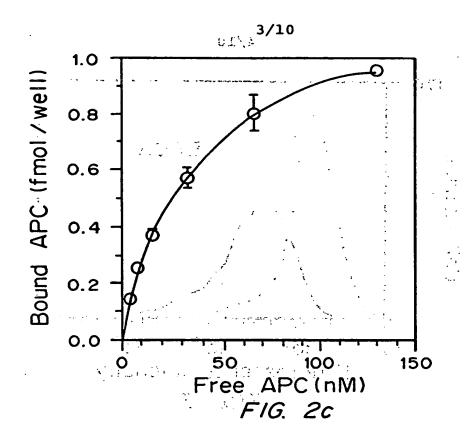


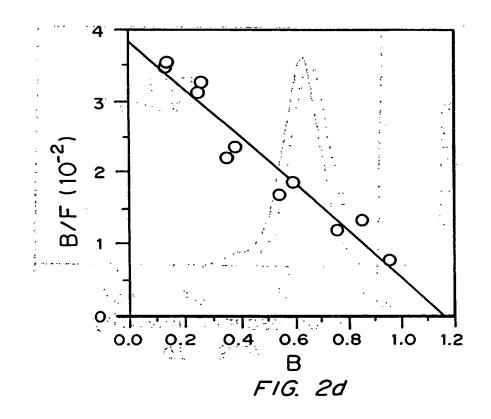




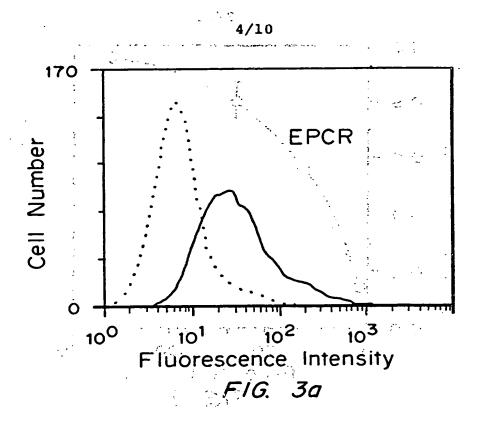


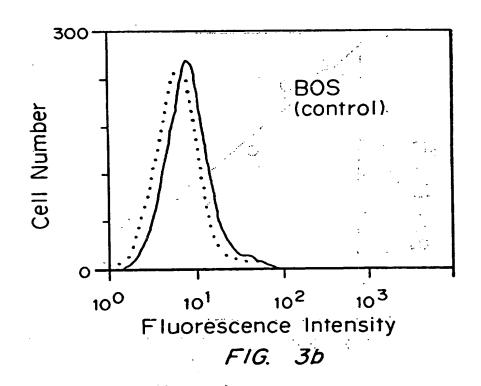
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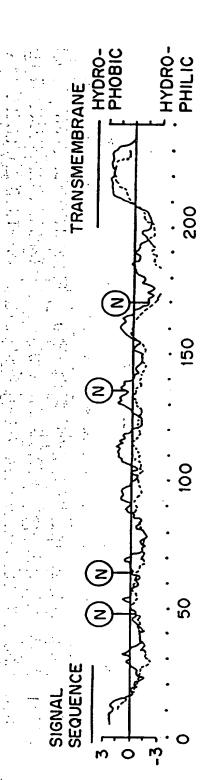
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1 MLTTLLPILLLSGWAFCSQDASDGLQRLHMLQISYFRDPYHVWYQGNA48					
1 MLTKFLPLLLLLPGCALCNSDGSQSLHMLQISYFQDHHHVRHQGNA 47					
49 SLGGHLTHVLEGPDTNTTIIQLQPLQEPESWARTQSGLQSYLLQFHGLVR 98	·*				
99 LVHQERTLAFPLTIRCFLGCELPPEGSRAHVFFEVAVNGSSFVSF 143					
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194 ISAENTKGSQTSRSYTSLVLGVLVGGFIIAGVAVGIFLCTGGRRC 238 . :					

MARIA COMMITTANATA

Internar | I Application No

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C. DOCUM	ENTS CONSIDERED	TO BE RELEVANT	· (1) 10 (1) 14 (1) 15 (1)	
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Y Furt	her documents are listed	in the continuation of box C.	Patent family members are listed in	ennex.
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* Special ca	tegones of cited docum	ents:	"I" later document published after the intern	ational filing date
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INTERNATIONAL CAREPORT

Interns ul Application No PCT/US 95/09636

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT			
Category *	Citation of document, with indication, where appropriate, of the relevant passages	\$ \frac{1}{2} \cdot \frac{1}{2	Relevant to claim No.
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification,		1-3,7-11
	cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document		
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I133		1-3,7-11
	K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel endothelial cell protein C/activated	e e e e e e e e e e e e e e e e e e e	
	see abstract 0707		
P,X	THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, September 1994 STUTTGART, GERMANY, pages 465-474, N. BANGALORE ET AL. High affinity binding sites for activated protein C and protein C on cultured human umbilical yein		1-3,7-11
	endothelial cells.' see summary see page 472, right column, line 9 - line 50		
P, X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 10 March 1995 BALTIMORE, MD, USA, pages 5571-5577, K. FUKUDOME ET AL. 'Molecular cloning and expression of murine and bovine		1-4,7-12
	endothelial cell protein C/activated protein C receptor (EPCR).	• • •	
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Intractional application No.

INTERNATIONAL SEARCH REPORT

PCT/US 95/09636

Box 1	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This int	ernational search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
ı. X	Claims Nos.: 16-18 because they relate to subject matter not required to be searched by this Authority, namely: Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.	Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This Inte	ernational Searching Authority found multiple inventions in this international application, as follows:
ı. 🗀	As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.	As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.	As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.	No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark o	The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.